PATENT
Atty. Docket No.: 8576.0067

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re U.S. Patent No. 5,840,299)
)
Issued: November 24, 1998)
)
To: Mary M. Bendig, Olivier J. Léger, José Saldanha,)
S. Tarran Jones, Ted A. Yednock)
)
Assignee: Athena Neurosciences, Inc.)
)
For: HUMANIZED ANTIBODIES AGAINST LEUKOCYTE)
ADHESION MOLECULE VLA-4)

ATTN: MAIL STOP PATENT EXT.
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

APPLICATION FOR EXTENSION OF PATENT
TERM UNDER 35 U.S.C. § 156

Applicant, Athena Neurosciences, Inc., represents that it is the Assignee of the entire interest in and to United States Patent No. 5,840,299 granted to Mary M. Bendig, Olivier J. Léger, José Saldanha, S. Tarran Jones, and Ted. A. Yednock on the 24th day of November 1998, for Humanized Antibodies Against Leukocyte Adhesion Molecule VLA-4 by virtue of an assignment from the inventors to Athena Neurosciences, Inc, recorded in the U.S. Patent and Trademark Office at Reel 7883, Frame 0465 on April 8, 1996. By the Power of Attorney enclosed herein (Attachment A), Applicant appoints several individual attorneys, including Charles E. Van Horn, as attorneys for Athena Neurosciences, Inc. with regard to this application for extension of the term of U.S.

APP I

Patent No. 5,840,299 and to transact all business in the U.S. Patent and Trademark Office in connection therewith.

Information Required Under 37 C.F.R. § 1.740

Applicant hereby submits this application for extension of the patent term under 35 U.S.C. § 156 by providing the following information required by the rules promulgated by the U.S. Patent and Trademark Office (37 C.F.R. § 1.740). For the convenience of the Patent and Trademark Office, the information contained in this application will be presented in a format which follows the requirements of Section 1.740 of Title 37 of the Code of Federal Regulations.

(1) The approved product TYSABRI® (natalizumab) is a recombinant humanized IgG4κ monoclonal antibody produced in murine myeloma cells. Natalizumab contains human framework regions and the complementarity-determining regions of a murine antibody that binds to α4-integrin. The molecular weight of natalizumab is 149 kilodaltons. TYSABRI® is supplied as a sterile, colorless, and clear to slightly opalescent concentrate for intravenous (IV) infusion.

(2) The approved product was subject to regulatory review under the Public Health Service Act.

(3) The approved product TYSABRI® received permission for commercial marketing or use under Section 351(a) of the Public Health Service Act on November 23, 2004. A copy of the FDA letter issuing Biologics License No. 1697 is attached (Attachment B).

(4) The active ingredient in TYSABRI® is natalizumab which, on information and belief, has not been approved for commercial marketing or use under the Public Health Services Act, the Virus-Serum-Toxin Act or under Section 505 of the Federal Food, Drug and Cosmetic Act prior to the issuance of Biologics License No. 1697 by the Food and Drug Administration on November 23, 2004. A copy of the package insert describing the approved product is attached (Attachment C).

(5) This application for extension of patent term under 35 U.S.C. § 156 is being submitted within the permitted 60-day period pursuant to 37 C.F.R. § 1.720(f), said period will expire on January 21, 2005.

(6) The complete identification of the patent for which a term extension is being sought is as follows:

Inventors: Mary M. Bendig, Olivier J. Léger, José Saldanha,
S. Tarran Jones, Ted A. Yednock

Patent No.: 5,840,299

Issue Date: November 24, 1998

Expiration Date: January 25, 2014

(7) A true copy of the patent is attached (Attachment D).

(8) No reexamination certificate or certificate of correction has been issued on this patent. A copy of a record of maintenance fee payments under 35 U.S.C. § 41(b) is attached (Attachment E).

(9) U.S. Patent No. 5,840,299 claims the active ingredient in TYSABRI®, a pharmaceutical composition comprising the active ingredient, and methods of using the active ingredient. The applicable patent claims are claims 1-7, 9, 11, 14, 18, 20, 23 and

25-29. The following description demonstrates the manner in which at least one claim reads on the active ingredient, a composition comprising the active ingredient, and a method of using the active ingredient.

(a) Claim 1 reads as follows: A humanized immunoglobulin comprising a humanized heavy chain and a humanized light chain: (1) the humanized light chain comprising three complementarity determining regions (CDR1, CDR2 and CDR3) having amino acid sequences from the corresponding complementarity determining regions of the mouse 21-6 immunoglobulin light chain variable domain designated SEQ. ID. No. 2, and a variable region framework from a human kappa light chain variable region framework sequence provided that at least one position selected from a first group consisting of L45, L49, L58 and L69 (Kabat numbering convention) is occupied by the same amino acid residue present in the equivalent position of the mouse 21-6 immunoglobulin light chain variable region framework; and (2) the humanized heavy chain comprising three complementarity determining regions (CDR1, CDR2 and CDR3) having amino acid sequences from the corresponding complementarity determining regions of the mouse 21-6 immunoglobulin heavy chain variable domain designated SEQ. ID. No. 4, and a variable region framework from a human heavy chain variable region framework sequence provided that at least one position selected from a second group consisting of H27, H28, H29, H30, H44, H71 (Kabat numbering convention) is occupied by the same amino acid residue present in the equivalent position of the mouse 21-6 immunoglobulin heavy chain variable region framework; wherein the humanized immunoglobulin specifically binds to

alpha-4-integrin with a binding affinity having a lower limit of about $10^7 M^{-1}$ and an upper limit of about five-times the binding affinity of the mouse 21-6 immunoglobulin wherein the 21-6 immunoglobulin has the light chain with a variable domain designated SEQ ID NO: 2 and IgG1 heavy chain with a variable domain designated SEQ ID NO: 4.

Claim 1 of the '299 patent reads on the active ingredient in TYSABRI®. The active ingredient meets all of the limitations of the claim. The active ingredient is a humanized immunoglobulin comprising both a humanized light chain and a humanized heavy chain. The sequence of the TYSABRI® light chain is provided in Figure 6 of the '299 patent (labeled as "La") and the sequence of the TYSABRI® heavy chain is provided in Figure 7 of the '299 patent (labeled as "Ha").

The light chain of the active ingredient contains three CDR regions (CDR1, CDR2, and CDR3). Each contain amino acids corresponding to the light chain CDR regions of the mouse 21-6 immunoglobulin (SEQ ID NO: 2 in the '299 patent and labeled in Figure 6 as "21.6") and a variable framework region from a human kappa light chain variable region framework sequence, with all four of the positions listed in the claim (L45, L49, L58, and L69) having the same amino acid residue as present in the corresponding position of the mouse antibody. The human kappa light chain variable region is provided in Figure 6 as "REI." Claim 9 of the '299 patent, which depends from claim 1, specifically recites that the antibody has the light chain sequence designated La in Figure 6.

The heavy chain of the active ingredient, similarly, contains three CDR regions (CDR1, CDR2, and CDR3). Each contain amino acids corresponding to the heavy chain CDR regions of the mouse 21-6 immunoglobulin (SEQ ID NO: 4 in the '299 patent and labeled in Figure 7 as "21.6") and a variable framework region from a human heavy chain variable region framework sequence, with five out of six of the positions listed in the claim (H27, H28, H29, H30, and H71) having the same amino acid residue as present in the corresponding position of the mouse antibody. The human heavy chain variable region is provided in Figure 7 as "2*CL." Claim 14 of the '299 patent, which depends from claim 9, specifically recites that the antibody has the heavy chain sequence designated Ha in Figure 7.

The active ingredient in TYSABRI®, likewise, meets the functional limitations of the claim. It specifically binds to alpha-4 integrin and has a binding affinity of 0.3 nM, which is between about $10^7 M^{-1}$ and 5 times the binding affinity of the mouse 21-6 immunoglobulin.

(b) Claim 2 reads as follows: The humanized immunoglobulin of claim 1 wherein the humanized light chain variable region framework is from an RE1 variable region framework sequence (SEQ. ID No:6) provided that at least one position is selected from the first group, and provided that at least one position selected from a third group consisting of positions L104, L105 and L107 (Kabat numbering convention) is occupied by the same amino acid residue present in the equivalent position of a kappa light chain from any human immunoglobulin other than RE1 (SEQ. ID No:6).

Claim 2 of the '299 patent also reads on the active ingredient in TYSABRI®. The active ingredient meets all of the limitations of the claim, as the light chain variable region framework is from an RE1 variable region framework sequence (SEQ ID NO. 6). See Figure 6. Claim 2 also requires that one position selected from the first group (described in claim 1) and that at least one position selected from a third group (L104, L105, and L107) is occupied by the same amino acid residue present in the equivalent position of a kappa light chain from any human antibody other than RE1. In the case of TYSABRI®, the three residues in the third group correspond to residues in the Campath antibody (another kappa light chain antibody). The analysis regarding claim 1 addressed the first group.

(c) Claim 3 reads as follows: The humanized immunoglobulin of claim 2, wherein the humanized heavy chain variable region framework is from a 21/28'CL variable region sequence (SEQ. ID No:10).

Claim 3 of the '299 patent also reads on the active ingredient in TYSABRI®. The active ingredient meets all of the limitations of the claim, as the humanized heavy chain variable region framework is from a 21/28'CL antibody. See Figure 6 and Brief Description of the Figures, col. 4, lines 17-28.

(d) Claim 4 reads as follows: The humanized immunoglobulin of claim 3, wherein the humanized light chain variable region framework comprises at least three amino acids from the mouse 21.6 immunoglobulin at positions in the first group and three amino acids from the kappa light chain from the human immunoglobulin other than REI at positions in the third group, and the

humanized heavy chain variable region framework comprises at least five amino acids from the mouse 21.6 immunoglobulin at positions in the second group.

Claim 4 of the '299 patent also reads on the active ingredient in TYSABRI®. The active ingredient meets all of the limitations of the claim. There are at least three amino acid residues from the mouse 21.6 antibody at the positions in the first group, as discussed above for claim 1. Additionally, there are at least three amino acids from another kappa light chain antibody in the third group, as discussed above for claim 2. Finally, there are at least five amino acids from the mouse 21.6 antibody at the positions in the second group, as discussed above for claim 1.

(e) Claim 5 reads as follows: The humanized immunoglobulin of claim 4, wherein the humanized light chain variable region framework is identical to the RE1 light chain variable region framework sequence except for the at least three positions from the first group and the three positions from the third group, and the heavy chain variable region framework is identical to the 21/28'CL heavy chain variable region framework sequence (SEQ. ID No:10) except for the at least five positions from the second group.

Claim 5 of the '299 patent also reads on the active ingredient in TYSABRI®. The active ingredient meets all of the limitations of the claim. The humanized light chain variable region framework is identical to the RE1 sequence except for the at least three positions from the first group (which includes the four differing positions in the first group of TYSABRI® due to the at least language in the claim) and three positions from the third group. The

humanized heavy chain variable region framework is identical to the 21/28'CL heavy chain variable region sequence except for the five positions from the second group.

(f) Claim 6 reads as follows: The humanized immunoglobulin of claim 5, wherein the at least three positions from the first group are positions L45, L58 and L69, and at the least five positions from the second group are positions H27, H28, H29, H30 and H71.

Claim 6 of the '299 patent also reads on the active ingredient in TYSABRI®. The active ingredient meets all of the limitations of the claim. The cited positions in the first and second groups have residues corresponding to the mouse 21-6 antibody, as discussed above for claim 1.

(g) Claim 7 reads as follows: The humanized immunoglobulin of claim 6, wherein the humanized light chain comprises complementarity determining regions that are identical to the corresponding complementarity determining regions of the mouse 21-6 heavy chain, and the humanized heavy chain comprises complementarity determining regions that are identical to the corresponding complementarity determining regions of the mouse 21-6 heavy chain, except that the CDR3 region of the humanized heavy chain may or may not comprise a phenylalanine residue at position H98.

Claim 7 of the '299 patent also reads on the active ingredient in TYSABRI®. The active ingredient meets all of the limitations of the claim, as the CDR's in both the heavy and light chains are identical to those in the mouse 21-6 antibody.

(h) Claim 9 reads as follows: The humanized immunoglobulin of claim 1, wherein the amino acid sequence of the mature light chain variable region is the sequence designated La (SEQ. ID NO:7) in FIG. 6 (SEQ ID NO:7).

Claim 9 of the '299 patent also reads on the active ingredient in TYSABRI®. The active ingredient meets all of the limitations of the claim. As discussed above for claim 1, La in Figure 6 represents the light chain variable region sequence for TYSABRI®.

(i) Claim 11 reads as follows: The humanized immunoglobulin of claim 1, wherein the amino acid sequence of the mature heavy chain variable region is the sequence designated Ha (SEQ ID NO:11) in FIG. 7 (SEQ ID NO: 11).

Claim 11 of the '299 patent also reads on the active ingredient in TYSABRI®. The active ingredient meets all of the limitations of the claim. As discussed above for claim 1, Ha in Figure 7 represents the heavy chain variable region sequence for TYSABRI®.

(j) Claim 14 reads as follows: The humanized immunoglobulin of claim 9, wherein the amino acid sequence of the mature heavy chain variable region is Ha (SEQ. ID NO:11) in FIG. 7 (SEQ ID NO: 11).

Claim 14 of the '299 patent also reads on the active ingredient in TYSABRI®. The active ingredient meets all of the limitations of the claim. As discussed above for claim 1, La in Figure 6 represents the light chain variable region sequence for TYSABRI® and Ha in Figure 7 represents the heavy chain variable region sequence for TYSABRI®.

(k) Claim 18 reads as follows: A humanized immunoglobulin of claim 14 or 16 that has a constant region domain.

Claim 18 of the '299 patent also reads on the active ingredient in TYSABRI®. The active ingredient meets all of the limitations of the claim, as the product has a constant region domain.

(l) Claim 20 reads as follows: The humanized immunoglobulin of claim 18, wherein the effector function is capable of complement fixation or antibody dependent cellular toxicity.

Claim 20 of the '299 patent also reads on the active ingredient in TYSABRI®. The antibody effector function of TYSABRI® is capable of at least one of complement fixation or antibody dependent cellular toxicity.

(m) Claim 23 reads as follows: A pharmaceutical composition comprising a humanized immunoglobulin of claim 14 or 16, or a binding fragment thereof, and a pharmaceutically acceptable carrier therefor.

Claim 23 likewise reads on a composition comprising the active ingredient in TYSABRI® since it may contain the humanized immunoglobulin of claim 14.

(n) Claim 25 reads as follows: A method of inhibiting adhesion of a leukocyte to an endothelial cell, the method comprising administering a therapeutically effective amount of the pharmaceutical composition of claim 23.

Claim 25 of the '299 patent also reads on a method of using the active ingredient in TYSABRI® since it uses the pharmaceutical composition of claim 23.

(o) Claim 26 reads as follows: The method of claim 25, wherein the endothelial cell is a brain cell.

Claim 26 of the '299 patent also reads on a method of using the active ingredient in TYSABRI® since it uses the pharmaceutical composition of claim 23.

(p) Claim 27 reads as follows: A method of treating an inflammatory disease in a patient comprising administering to the patient a therapeutically effective amount of the pharmaceutical composition of claim 23.

Claim 27 reads on a method of using the active ingredient in TYSABRI® since it uses the pharmaceutical composition of claim 23.

(q) Claim 28 reads as follows: The method of claim 27 wherein the inflammatory disease is multiple sclerosis.

Claim 28 reads on a method of using the active ingredient in TYSABRI® since it uses the pharmaceutical composition of claim 23.

(r) Claim 29 reads as follows: The method of claim 27, wherein the patient is already suffering from multiple sclerosis and the administration of the pharmaceutical composition at least partially arrests the symptoms of the disease.

Claim 29 reads on a method of using the active ingredient in TYSABRI® since it uses the pharmaceutical composition of claim 23.

(10) The relevant dates and information pursuant to 35 U.S.C. § 156(g) to enable the Secretary of Health and Human Services to determine the applicable regulatory review period are as follows:

Investigational New Drug Application (BB-IND 6895) for TYSABRI® was filed on October 23, 1996 and became effective on November 23, 1996.

Original Biologics Licensing Application for TYSABRI® (BLA 125104) was submitted on May 24, 2004.

Biologics License No. 1697 for TYSABRI® was issued on November 23, 2004.

(11) A brief description of the significant activities undertaken by the marketing applicant during the applicable regulatory review period with respect to TYSABRI® and the dates applicable to these significant activities are set forth in a chronology of events in Attachment F.

Authorizations by Biogen Idec Inc. and Elan Pharmaceuticals, Inc. for applicant, Athena Neurosciences, Inc., to rely on the regulatory activities for TYSABRI® are contained in Attachment G.

(12)(i) Applicant is of the opinion that U.S. Patent No. 5,840,299 is eligible for extension of the patent term under 35 U.S.C. § 156 because it satisfies all requirements for such extension as follows:

(a) 35 U.S.C. § 156(a) - U.S. Patent No. 5,840,299 claims a humanized immunoglobulin (the active ingredient in TYSABRI®), a pharmaceutical composition comprising the active ingredient, and methods of using the active ingredient.

(b) 35 U.S.C. § 156(a)(1) - U.S. Patent No. 5,840,299 has not expired before submission of this application.

(c) 35 U.S.C. § 156(a)(2) - The term of U.S. Patent No. 5,840,299 has never been extended under 35 U.S.C. § 156(e)(1).

(d) 35 U.S.C. § 156(a)(3) - The application for patent term extension is submitted by the owner of record of the patent in accordance with the requirements of paragraphs (1) through (4) of 35 U.S.C. § 156(d) and the rules of the Patent and Trademark Office.

(e) 35 U.S.C. § 156(a)(4) - The product TYSABRI® has been subjected to a regulatory review period before its commercial marketing or use.

(f) 35 U.S.C. § 156(a)(5)(A) - The commercial marketing or use of the product TYSABRI® after the regulatory review period is the first permitted commercial marketing or use under the provisions of § 351(a) of the Public Health Service Act under which such regulatory review period occurred.

(g) 35 U.S.C. § 156(c)(4) - No other patent has been extended for the same regulatory review period for the product TYSABRI®.

(12)(ii) Applicant respectfully submits that the length of the extension of patent term for U.S. Patent No. 5,840,299 is 1189 days pursuant to 35 U.S.C. § 156(c). The length of the extension was determined pursuant to 37 C.F.R. § 1.775 as follows:

(a) The regulatory review period under 35 U.S.C. § 156(g)(1)(B) began on November 23, 1996 and ended November 23, 2004, which is a total of 2924 days, which is the sum of (1) and (2) below:

(1) The period of review under 35 U.S.C. § 156(g)(1)(B)(i), the "Testing Period," began on November 23, 1996 and ended on May 24, 2004, which is 2740 days; and

(2) The period of review under 35 U.S.C. § 156(g)(1)(B)(ii), the "Approval Period," began on May 24, 2004, and ended on November 23, 2004, which is a total of 184 days.

(b) The regulatory review period upon which the period of extension is calculated is the entire regulatory review period as determined in subparagraph 12(ii)(a) above (2924) less:

(1) The number of days in the regulatory review period which were on or before the date on which the patent issued (November 24, 1998) which is 731 days; and

(2) The number of days during which applicant did not act with due diligence, which is zero (0) days; and

(3) One-half the number of days determined in subparagraph (12)(ii)(a)(1) above after the patent issued (one-half of 2009) which is 1005 days;

(c) The number of days as determined in subparagraph (12)(ii)(b) (1189 days) when added to the original term of the patent (January 25, 2014) would result in the date of April 28, 2017.

(d) Fourteen (14) years when added to the date of issuance of the Biologics License (November 23, 2004) would result in the date of November 23, 2018;

(e) The earlier date as determined in subparagraphs (12)(ii)(c) and (12)(ii)(d) is April 28, 2017;

(f) Since U.S. Patent No. 5,840,299 issued after September 24, 1984, the period of extension may not exceed five years from the original expiration date of January 25, 2014. Five years when added to the original expiration date of the patent would result in the date of January 25, 2019.

(g) The earlier date as determined by subparagraphs (12)(ii)(e) and (12)(ii)(f) is April 28, 2017.

(13) Applicant acknowledges a duty to disclose to the Director of the United States Patent and Trademark Office and the Secretary of Health and Human Services any information which is material to the determination of entitlement to the extension sought.

(14) The prescribed fee for receiving and acting upon this application is attached as a check in the amount of $1,120.00. The Director is authorized to charge any additional fees required by this application to Deposit Account No. 06-0916.

(15) All correspondence and inquiries may be directed to the undersigned, whose address, telephone number and fax number are as follows:

Charles E. Van Horn
Finnegan, Henderson, Farabow, Garrett & Dunner, L.L.P.
901 New York Avenue, N.W.
Washington, D.C. 20001-4413
Phone: 202-408-4000
Fax: 202-408-4400

(16) Enclosed is a certification that the application for extension of patent term under 35 U.S.C. § 156 including its attachments and supporting papers is being submitted as one original and two (2) copies thereof (Attachment H).

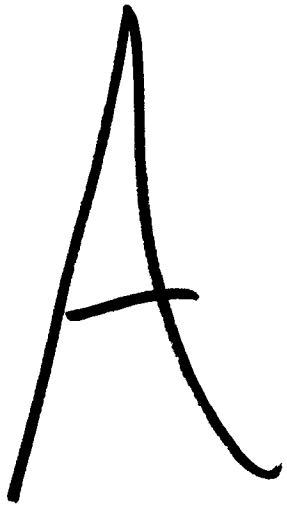
Respectfully submitted,

FINNEGAN, HENDERSON, FARABOW,
GARRETT & DUNNER, L.L.P.

By: Charles E. Van Horn
Charles E. Van Horn
Reg. No. 40,266

Date: January 18, 2005

Attachments:
Power of Attorney (Attachment A)
Approval Letter (Attachment B)
Package Insert for TYSABRI® (Attachment C)
U.S. Patent No. 5,840,299 (Attachment D)
Maintenance Fees Paid (Attachment E)
Chronology of Regulatory Review Period (Attachment F)
Authorizations for Athena Neurosciences, Inc. to Rely on Regulatory Review of TYSABRI® (Attachment G)
Certification of Copies of Application Papers (Attachment H)



PATENT
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In re U.S. Patent No. 5,840,299)
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To: Mary M. Bendig, Olivier J. Léger, José Saldanha,)
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Assignee: Athena Neurosciences, Inc.)
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For: HUMANIZED ANTIBODIES AGAINST LEUKOCYTE)
ADHESION MOLECULE VLA-4)

ATTN: MAIL STOP PATENT EXT. **Attachment A**
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

POWER OF ATTORNEY BY ASSIGNEE

The undersigned, a representative authorized to sign on behalf of the assignee owning all of the interest in this patent, verifies that Athena Neurosciences, Inc. is the assignee of the entire right, title, and interest in U.S. Patent No. 5,840,299 (the '299 patent) by virtue of an assignment from the inventors to Athena Neurosciences, Inc., recorded in the U.S. Patent and Trademark Office at Reel 7883, Frame 0465 on April 8, 1996. To the best of the undersigned's knowledge and belief, title to the '299 patent is in the assignee.

The undersigned hereby grants power of attorney to

Charles E. Van Horn, Reg. No. 40,266

James B. Monroe, Reg. No. 33,971

Leslie Boley, Reg. No. 41,490

Nina Ashton, Reg. No. 37,273

Mark Hoch, Reg. No. 35,195

Carl Battle, Reg. No. 30,731

Richard Hake, Reg. No. 37,343

both jointly and separately as attorneys with full power of substitution and revocation to prosecute the application for patent term extension of the '299 patent and to transact all business in the Patent and Trademark Office connected therewith.

Please send all future correspondence concerning this application for patent term extension to Finnegan, Henderson, Farabow, Garrett & Dunner, L.L.P. at the following address:

Finnegan, Henderson, Farabow,
Garrett & Dunner, L.L.P.
901 New York Avenue, N.W.
Washington, D.C. 20001-4413

Dated: January 12, 2005

By: _____

Name: Carl W. Battle

Title: Vice President

Assignee: ATHENA NEUROSCIENCES, INC.

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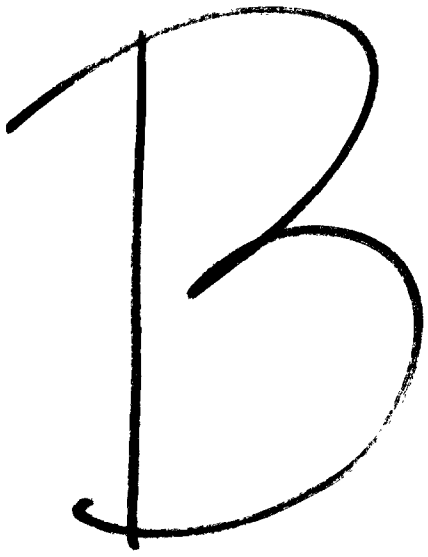


Attachment B

DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service

Food and Drug Administration
Rockville, MD 20852

Our STN: BL 125104/0

NOV 23 2004

Biogen Idec Inc.
Attention: Nadine D. Cohen, Ph.D.
Senior Vice President, Regulatory Affairs
14 Cambridge Center
Cambridge, MA 02142

Dear Dr. Cohen:

We are issuing Department of Health and Human Services U.S. License No. 1697 to Biogen Idec Inc., Cambridge, Massachusetts, under the provisions of section 351(a) of the Public Health Service Act controlling the manufacture and sale of biological products. The license authorizes you to introduce or deliver for introduction into interstate commerce, those products for which your company has demonstrated compliance with establishment and product standards.

Under this license, you are authorized to manufacture the product Natalizumab. Natalizumab is indicated for the treatment of patients with relapsing forms of multiple sclerosis to reduce the frequency of clinical exacerbations.

Under this license, you are approved to manufacture Natalizumab drug substance at Biogen Idec, Research Triangle Park, North Carolina. The final formulated product will be manufactured and filled at (b)(4) , and labeled and packaged at (b)(4) (b)(4) You may label your product with the proprietary name TYSABRI® and will market it in a 15 mL vial containing 300 mg (20 mg/mL).

The dating period for Natalizumab shall be 15 months from the date of manufacture when stored at 2°-8°C (36°-46°F). The date of manufacture shall be defined as the date of final (b)(4) of the formulated drug product. The dating period for your drug substance shall be 15 months when stored at 2°-8°C (36°-46°F). We have approved the stability protocol in your license application for the purpose of extending the expiration dating period of your drug substance and drug product under 21 CFR 601.12.

You currently are not required to submit samples of future lots of Natalizumab to the Center for Drug Evaluation and Research (CDER) for release by the Director, CDER, under 21 CFR 610.2. We will continue to monitor compliance with 21 CFR 610.1 requiring completion of tests for conformity with standards applicable to each product prior to release of each lot.

You must submit information to your biologics license application (BLA) for our review and written approval under 21 CFR 601.12 for any changes in the manufacturing, testing, packaging or labeling of Natalizumab, or in the manufacturing facilities.

All applications for new active ingredients, new dosage forms, new indications, new routes of administration, and new dosing regimens are required to contain an assessment of the safety and effectiveness of the product in pediatric patients unless this requirement is waived or deferred. We reference the full waiver granted on August 2, 2002, for the pediatric study requirement for this application, in accordance with 21 CFR.601.27(c)(2)(ii).

As requested in your letter of September 13, 2004, marketing approval of this product is granted under the accelerated approval of biological products regulations, 21 CFR 601.40-46. These regulations permit the use of certain surrogate endpoints or an effect on a clinical endpoint other than survival or irreversible morbidity as basis for approvals of products intended for serious or life-threatening illnesses or conditions.

Approval under these regulations requires, among other things, that you conduct adequate and well-controlled studies to verify and describe the clinical benefit attributable to this product. Clinical benefit is evidenced by effects such as increased survival or improvement in disease-related symptoms. You are required to conduct such studies with due diligence. If postmarketing studies fail to verify that clinical benefit is conferred by Natalizumab, or are not conducted with due diligence, the Agency may, following a hearing, withdraw or modify approval.

Granting of this approval is contingent upon completion of clinical studies to verify the clinical benefit of Natalizumab therapy, as outlined in your letters of November 19 and November 23, 2004. The postmarketing study is subject to the reporting requirements of 21 CRF 601.70:

1. To verify that the clinical benefit of reduction in exacerbations is sustained with continued Natalizumab administration by completing the ongoing Protocols C-1801 and C-1802, "A Randomized, Double-blind, Placebo-Controlled, Parallel-Group, Multicenter Study to Determine the Safety and Efficacy of Natalizumab in Subjects with Relapsing-Remitting Multiple Sclerosis" and "A Randomized, Double-Blind, Placebo-Controlled, Parallel-Group, Multicenter Study to Determine the Safety and Efficacy of Natalizumab, When Added to Avonex® (Interferon beta-1a) in Subjects with Relapsing-Remitting Multiple Sclerosis" through the planned two years and to submit the results along with the appropriate label changes. The final protocols were submitted on September 16, 2003 (C-1801 and C-1802). Accrual was completed on July 31, 2002 (C-1801) and January 15, 2003 (C-1802). The studies will be completed by December 1, 2004, (C-1801) and March 31, 2005 (C-1802) and the final study reports and revised labeling will be submitted by September 30, 2005.

For administrative purposes, all submissions related to these postmarketing study commitments should be clearly designated "Subpart E Postmarketing Study Commitments."

In addition, we acknowledge your written commitments as described in your letters of November 19 and November 23, 2004, as outlined below:

Additional Postmarketing Studies subject to reporting requirements of 21 CFR 601.70 Studies

2. To further evaluate the safety of Natalizumab and the efficacy of Natalizumab on physical disability by completing the ongoing Protocols C-1801 and C-1802, "A Randomized, Double-Blind, Placebo-Controlled, Parallel-Group, Multicenter Study to Determine the Safety and Efficacy of Natalizumab in Subjects with Relapsing-Remitting Multiple Sclerosis" and "A Randomized, Double-Blind, Placebo-Controlled, Parallel-Group, Multicenter Study to Determine the Safety and Efficacy of Natalizumab, When Added to Avonex® (Interferon beta-1a) in Subjects with Relapsing-Remitting Multiple Sclerosis" through the planned two years and to submit the results along with the appropriate label changes. The final protocols were submitted on September 16, 2003 (C-1801 and C-1802). Accrual was completed on July 31, 2002 (C-1801) and January 15, 2003 (C-1802). The studies will be completed by December 1, 2004 (C-1801) and March 31, 2005 (C-1802) and the final study reports and revised labeling will be submitted by September 30, 2005.

3. To conduct intensive pharmacokinetic samplings of at least six months duration as part of Study C-1808, "An Open-label, Multicenter Extension Study to Evaluate the Safety and Tolerability of Natalizumab in Subjects with Multiple Sclerosis Who Have Completed Studies C-1801, C-1802, or C-1803," to determine the pharmacokinetics of chronically dosed Natalizumab in the presence of glatiramer acetate. The final protocol will be amended and submitted by March 31, 2005 accrual will be completed by December 31, 2005, study completion will occur by January 31, 2006 and the final study report will be submitted by October 31, 2006.

4. To conduct a Pregnancy Registry for Natalizumab to evaluate approximately 300 Natalizumab-exposed pregnancies for patterns or increases in birth defects in children of women with multiple sclerosis who are exposed to Natalizumab at the time of conception, or at any time during pregnancy. The protocol will include a concurrent control group, to assess birth defects in children of women with multiple sclerosis who are not exposed to Natalizumab prior to conception or during pregnancy. The final protocol will be submitted by June 30, 2005. Accrual will be completed by July 31, 2013. The study will be completed by July 31, 2014 and the final study report will be submitted by April 30, 2015.

5. To develop an immunogenicity screening assay that is less susceptible to interference by circulating drug. In the event that a suitable assay format that meets requirements is identified, the assay will be further developed and validated by June 30, 2006, for the detection of anti-Natalizumab specific antibodies. The final study report will be submitted by March 31, 2007.

6. To further optimize the existing assay or develop a new assay for the detection of neutralizing antibodies. Following optimization of assay parameters, the assay negative cut-point will be redefined and the assay revalidated. Assay validation will be completed by December 31, 2005. The final study report will be submitted by September 30, 2006.

7. To conduct a study to measure the effects of Natalizumab on percentages of lymphocytes including CD3+, CD4+, and CD8+ as well as B and NK cells and the associated α4 integrin expression and binding site saturation. The final protocol will be submitted by June 30, 2005. Accrual will be completed by December 15, 2006. The study will be completed by June 15, 2007 and the final study report will be submitted by March 15, 2008.

8. To conduct a single-center, multiple dose study to measure the effects of Natalizumab on responses to neo-antigen and recall vaccination in subjects with relapsing multiple sclerosis in approximately 40 subjects who have a series of two booster immunizations. The final protocol will be submitted by June 30, 2005. Accrual will be completed by December 15, 2006. The study will be completed by June 15, 2007, and the final study report will be submitted by March 15, 2008.

9. To develop an assay to quantify bispecific Natalizumab IgG4 antibodies in human serum samples. Reagent development feasibility studies will be completed by March 31, 2006, and if successful, an assay for detecting bispecific Natalizumab IgG4 antibodies will be developed and validated by June 30, 2006. The final study report will be submitted by March 31, 2007.

10. To test the samples obtained from the pharmacokinetic sampling cohort in Studies C-1803 and C-1808 for bispecific Natalizumab IgG4 antibodies, should a sensitive assay for Natalizumab IgG4 antibodies be developed. The final protocol for Study C-1803 was submitted on November 26, 2003. The final protocol for Study C-1808 will be amended and submitted by March 31, 2005. Accrual was completed for Study C-1803 on October 7, 2003 and will be completed for Study C-1808 by March 31, 2005. The study will be completed by June 30, 2007 and the final report will be submitted by March 31, 2008.

11. To use the current screening assay to assess the immunogenicity of Natalizumab by conducting a study of patients who are at least three months post-treatment. This commitment will be addressed as part of Study C-1808, "An Open-label, Multicenter Extension Study to Evaluate the Safety and Tolerability of Natalizumab in Subjects with Multiple Sclerosis Who Have Completed Studies C-1801, C-1802, or C-1803." The final protocol will be amended and submitted by March 31, 2005. Accrual will be completed by March 31, 2005. The study will completed by March 29, 2007. The final study report will be submitted by October 31, 2007.

12. To retest all available samples from completed studies C-1801 and C-1802 once new screening and neutralizing immunogenicity assays have been successfully developed (see commitments 5 and 6). The final protocols were submitted on September 16, 2003 (C-1801 and C-1802). Accrual was completed on July 31, 2002 (C-1801) and January 15, 2003 (C-1802). The study will be completed by December 31, 2006. The final study report will be submitted by June 30, 2007.

Postmarketing Studies not subject to reporting requirements of 21 CFR 601.70.

13. To conduct a study measuring the effects of freeze/thaw on Natalizumab drug product. All drug product release tests will be performed. The final study report will be submitted by June 30, 2005.

14. To conduct a study to detect and quantify (b)(4) that could be present in (b)(4) of Natalizumab drug product. The study can use a placebo formulation if this facilitates the detection of (b)(4) . The study will identify and assess worst-case stability/shipping conditions in which (b)(4) could be found in Natalizumab drug product. The final study report will be submitted by June 30, 2005.

15. To re-evaluate drug substance and drug product release and in-process specifications after the first 30 lots of each are produced. Specifications and limits will be adjusted, if necessary. The data and analysis for the first 30 lots will be provided in the 2006 Annual Report to be submitted by January 31, 2007.

16. To submit the final study report for Study #309-33-01 for the prenatal and postnatal development study of Natalizumab in cynomolgus monkeys by March 31, 2005.

We request that you submit clinical protocols to your IND, BB-IND 6895, with a cross-reference letter to this BLA, STN BL 125104. Submit nonclinical and chemistry, manufacturing, and controls protocols and all study final reports to your BLA, STN BL125104. Please use the following designators to label prominently all submissions, including supplements, relating to these postmarketing study commitments as appropriate:

- **Postmarketing Study Protocol**
- **Postmarketing Study Final Report**
- **Postmarketing Study Correspondence**
- **Annual Report on Postmarketing Studies**

For each postmarketing study subject to the reporting requirements of 21 CFR 601.70, you must describe the status in an annual report on postmarketing studies for this product. The status report for each study should include:

- information to identify and describe the postmarketing commitment,
- the original schedule for the commitment,

- the status of the commitment (i.e., pending, ongoing, delayed, terminated, or submitted),
- an explanation of the status including, for clinical studies, the patient accrual rate (i.e., number enrolled to date and the total planned enrollment), and
- a revised schedule if the study schedule has changed and an explanation of the basis for the revision.

As described in 21 CFR 601.70(e), we may publicly disclose information regarding these postmarketing studies on our Web site (http://www.fda.gov/cder/pmc/default.htm). Please refer to the April 2001 Draft Guidance for Industry: Reports on the Status of Postmarketing Studies - Implementation of Section 130 of the Food and Drug Administration Modernization Act of 1997 (see http://www.fda.gov/cber/gdlns/post040401.htm) for further information.

As required by 21 CFR 601.45, submit all promotional materials at least 30 days before the intended time of initial distribution of labeling or initial publication of the advertisement with a cover letter requesting advisory comment. Send two copies of the promotional materials to the Division of Drug Marketing, Advertising and Communications, HFD-42, Food and Drug Administration, 5600 Fishers Lane, Rockville MD 20852. Please submit final promotional materials with FDA Form 2253 to the above address at the time of initial dissemination of the labeling or at the time of initial publication of the advertisement.

All promotional claims must be consistent with and not contrary to approved labeling. You should not make a comparative promotional claim or claim of superiority over other products unless you have substantial evidence to support that claim.

You must submit adverse experience reports under the adverse experience reporting requirements for licensed biological products (21 CFR 600.80). You should submit postmarketing adverse experience reports to the Central Document Room, Center for Drug Evaluation and Research, Food and Drug Administration, 5901-B Ammendale Road, Beltsville, MD 20705-1266. Prominently identify all adverse experience reports as described in 21 CFR 600.80.

The MedWatch-to-Manufacturer Program provides manufacturers with copies of serious adverse event reports that are received directly by the FDA. New molecular entities and important new biologics qualify for inclusion for three years after approval. Your firm is eligible to receive copies of reports for this product. To participate in the program, please see the enrollment instructions and program description details at www.fda.gov/medwatch/report/mmp.htm.

You must submit distribution reports under the distribution reporting requirements for licensed biological products (21 CFR 600.81).

You must submit reports of biological product deviations under 21 CFR 600.14. You should promptly identify and investigate all manufacturing deviations, including those associated with processing, testing, packing, labeling, storage, holding and distribution. If the deviation

involves a distributed product, may affect the safety, purity, or potency of the product, and meets the other criteria in the regulation, you must submit a report on Form FDA-3486 to the Division of Compliance Risk Management and Surveillance (HFD-330), Center for Drug Evaluation and Research, Food and Drug Administration, 5600 Fishers Lane, Rockville, MD 20857.

Please submit all final printed labeling at the time of use and include implementation information on FDA Form 356h. Please provide a PDF-format electronic copy as well as original paper copies (ten for circulars and five for other labels).

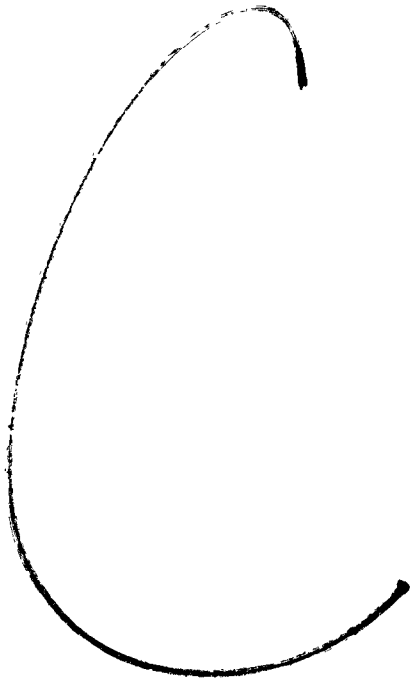
Please refer to http://www.fda.gov/cder/biologics/default.htm for important information regarding therapeutic biological products, including the addresses for submissions. Effective Oct. 4, 2004, the new address for all submissions to this application is:

CDER Therapeutic Biological Products Document Room
Center for Drug Evaluation and Research
Food and Drug Administration
12229 Wilkins Avenue
Rockville, Maryland 20852

Sincerely,

(b)(6)

Karen D. Weiss, M.D.
Director
Office of Drug Evaluation VI
Center for Drug Evaluation and Research



TYSABRI® (natalizumab)

DESCRIPTION

TYSABRI® (natalizumab) is a recombinant humanized IgG4κ monoclonal antibody produced in murine myeloma cells. Natalizumab contains human framework regions and the complementarity-determining regions of a murine antibody that binds to α4-integrin. The molecular weight of natalizumab is 149 kilodaltons. TYSABRI® is supplied as a sterile, colorless, and clear to slightly opalescent concentrate for intravenous (IV) infusion.

Each 15 mL dose contains 300 mg natalizumab; 123 mg sodium chloride, USP; 17.0 mg sodium phosphate, monobasic, monohydrate, USP; 7.24 mg sodium phosphate, dibasic, heptahydrate, USP; 3.0 mg polysorbate 80, USP/NF, in water for injection, USP at pH 6.1.

CLINICAL PHARMACOLOGY

General

TYSABRI® binds to the α4-subunit of α4β1 and α4β7 integrins expressed on the surface of all leukocytes except neutrophils, and inhibits the α4-mediated adhesion of leukocytes to their counter-receptor(s). The receptors for the α4 family of integrins include vascular cell adhesion molecule-1 (VCAM-1), which is expressed on activated vascular endothelium, and mucosal addressin cell adhesion molecule-1 (MadCAM-1) present on vascular endothelial cells of the gastrointestinal tract. Disruption of these molecular interactions prevents transmigration of leukocytes across the endothelium into inflamed parenchymal tissue. *In vitro*, anti-α4-integrin antibodies also block α4-mediated cell binding to ligands such as osteopontin and an alternatively spliced domain of fibronectin, connecting segment-1 (CS-1). *In vivo*, TYSABRI® may further act to inhibit the interaction of α4-expressing leukocytes with their ligand(s) in the extracellular matrix and on parenchymal cells, thereby inhibiting further recruitment and inflammatory activity of activated immune cells.

The specific mechanism(s) by which TYSABRI® exerts its effects in multiple sclerosis have not been fully defined. In multiple sclerosis, lesions are believed to occur when activated inflammatory cells, including T-lymphocytes, cross the blood-brain barrier (BBB). Leukocyte migration across the BBB involves interaction between adhesion molecules on inflammatory cells, and their counter-receptors present on endothelial cells of the vessel wall. The clinical effect of natalizumab in multiple sclerosis may be secondary to blockade of the molecular interaction of α4β1-integrin expressed by inflammatory cells with VCAM-1 on vascular endothelial cells, and with CS-1 and/or osteopontin expressed by parenchymal cells in the brain. Data from an experimental autoimmune encephalitis animal model of multiple sclerosis demonstrate reduction of leukocyte migration into brain parenchyma and reduction of plaque formation detected by magnetic resonance imaging (MRI) following repeated administration of natalizumab. The clinical significance of these animal data is unknown.

Pharmacokinetics

Following the repeat intravenous administration of a 300 mg dose of natalizumab to multiple sclerosis

patients, the mean maximum observed serum concentration was 98 ± 34 mcg/mL. Mean average steady-state natalizumab concentrations over the dosing period were approximately 30 mcg/mL. The mean half-life of 11 ± 4 days was observed with a clearance of 16 ± 5 mL/hour. The distribution volume of 5.7 ± 1.9 L was consistent with plasma volume.

Pharmacokinetics of TYSABRI® in pediatric multiple sclerosis patients or patients with renal or hepatic insufficiency have not been studied.

Pharmacodynamics

TYSABRI® administration increases the number of circulating leukocytes, (including lymphocytes, monocytes, basophils, and eosinophils) due to inhibition of transmigration out of the vascular space. TYSABRI® does not affect the number of circulating neutrophils **(see PRECAUTIONS, Laboratory Tests)**.

CLINICAL STUDIES

TYSABRI® was evaluated in two ongoing randomized, double-blind, placebo-controlled trials in patients with multiple sclerosis. Both studies enrolled patients who experienced at least one clinical relapse during the prior year and had a Kurtzke Expanded Disability Status Scale (EDSS) score between 0 and 5.0.

In both studies, neurological evaluations were performed every 12 weeks and at times of suspected relapse. Magnetic resonance imaging evaluations for T1-weighted gadolinium (Gd)-enhancing lesions and T2-hyperintense lesions were performed annually.

Study 1 enrolled patients who had not received any interferon-beta or glatiramer acetate for at least the previous 6 months; approximately 94% had never been treated with these agents. Median age was 37, with a median disease duration of 5 years. Patients were randomized in a 2:1 ratio to receive TYSABRI® 300 mg IV infusion (n=627) or placebo (n=315) every 4 weeks for up to 28 months.

Study 2 enrolled patients who had experienced one or more relapses while on treatment with AVONEX® (Interferon beta-1a) 30 mcg intramuscularly (IM) once weekly during the year prior to study entry. Median age was 39, with a median disease duration of 7 years. Patients were evenly randomized to receive TYSABRI® 300 mg (n=589) or placebo (n=582) every 4 weeks for up to 28 months. All patients continued to receive AVONEX® 30 mcg IM once weekly.

Results for each study were analyzed at a pre-specified time and are shown in Tables 1 and 2. Median patient time on study was 13 months in both studies. Safety and efficacy of treatment with TYSABRI® beyond one year are not known.

The exact relationship between MRI findings and the clinical status of patients is unknown. Changes in lesion area often do not correlate with changes in disability progression. The prognostic significance of the MRI findings in these studies has not been evaluated.

Table 1. 13-Month Clinical and 1-Year MRI Endpoints in Study 1 (Monotherapy Study)

	TYSABRI® n=627	**Placebo** n=315
Clinical Endpoints		
Annualized relapse rate	0.25	0.74
Relative reduction (percentage)	66%	
Percentage of patients remaining relapse-free	76%	53%
MRI Endpoints		
New or newly enlarging T2-hyperintense lesions		
Median	0.0	3.0
Percentage of patients with:		
0 lesions	60%	22%
1 lesion	18%	13%
2 lesions	6%	7%
3 or more lesions	16%	58%
Gd-enhancing lesions		
Median	0.0	0.0
Percentage of patients with:		
0 lesions	96%	68%
1 lesion	3%	13%
2 or more lesions	1%	19%

All analyses were intent-to-treat. For each endpoint, $p<0.001$ Determination of p-values: relapse rate by Poisson regression adjusting for baseline relapse rate, EDSS, presence of Gd-enhancing lesions, age, percentage relapse-free by logistic regression adjusting for baseline relapse rate; and MRI endpoints by ordinal logistic regression adjusting for baseline lesion number

Table 2. 13-Month Clinical and 1-Year MRI Endpoints in Study 2 (Add-On Study)

	TYSABRI® plus AVONEX® n=589	**Placebo plus AVONEX®** n=582
Clinical Endpoints		
Annualized relapse rate	0.36	0.78
Relative reduction (percentage)	54%	
Percentage of patients remaining relapse-free	67%	46%

	TYSABRI® plus AVONEX® n=589	Placebo plus AVONEX® n=582
MRI Endpoints		
New or newly enlarging T2-hyperintense lesions		
Median	0.0	1.0
Percentage of patients with:		
0 lesions	67%	40%
1 lesion	26%	29%
2 lesions	4%	10%
3 or more lesions	3%	21%
Gd-enhancing lesions		
Median	0.0	0.0
Percentage of patients with:		
0 lesions	96%	76%
1 lesion	3%	12%
2 or more lesions	1%	12%

All analyses were intent-to-treat. For each endpoint, $p<0.001$. Determination of p-values. relapse rate by Poisson regression adjusting for baseline relapse rate, EDSS, presence of Gd-enhancing lesions, age; percentage relapse-free by logistic regression adjusting for baseline relapse rate; and MRI endpoints by ordinal logistic regression adjusting for baseline lesion number.

INDICATIONS AND USAGE

TYSABRI® is indicated for the treatment of patients with relapsing forms of multiple sclerosis to reduce the frequency of clinical exacerbations. This indication is based on results achieved after approximately one year of treatment in ongoing controlled trials of two years in duration. The safety and efficacy of TYSABRI® beyond one year are unknown.

Safety and efficacy in patients with chronic progressive multiple sclerosis have not been established.

CONTRAINDICATIONS

TYSABRI® should not be administered to patients with known hypersensitivity to TYSABRI® or any of its components.

WARNINGS

Hypersensitivity

TYSABRI® has been associated with hypersensitivity reactions, including serious systemic reactions (e.g., anaphylaxis) which occurred at an incidence of <1%. These reactions usually occur within 2 hours of the start of the infusion. Symptoms associated with these reactions can include urticaria, dizziness, fever, rash, rigors, pruritus, nausea, flushing, hypotension, dyspnea, and chest pain. Generally, these reactions are associated with antibodies to TYSABRI®.

If a hypersensitivity reaction occurs, discontinue administration of TYSABRI® and initiate appropriate therapy **(see ADVERSE REACTIONS, Infusion-related Reactions)**. Patients who have experienced a hypersensitivity reaction should not be re-treated with TYSABRI®. The possibility of antibodies to TYSABRI® should be considered in patients who have hypersensitivity reactions **(see ADVERSE REACTIONS, Immunogenicity)**.

PRECAUTIONS

Immunosuppression

In Studies 1 and 2, concomitant treatment of relapses with a short course of corticosteroids was not associated with an increased rate of infection. The safety and efficacy of TYSABRI® in combination with other immunosuppressive agents have not been evaluated. Patients receiving these agents should not receive concurrent therapy with TYSABRI® because of the possibility of increased risk of infections.

Information to Patients

If patients experience symptoms consistent with a hypersensitivity reaction (e.g., urticaria with or without associated symptoms) during or following an infusion of TYSABRI®, they should report these symptoms to their physician immediately **(see WARNINGS, Hypersensitivity)**.

Laboratory Tests

TYSABRI® induces increases in circulating lymphocytes, monocytes, eosinophils, basophils, and nucleated red blood cells. Observed increases persist during TYSABRI® exposure, but are reversible, returning to baseline levels usually within 16 weeks after the last dose. Elevations of neutrophils are not observed.

Drug Interactions

After multiple dosing, interferon beta-1a (AVONEX® 30 mcg IM once weekly) reduced TYSABRI® clearance by approximately 30%. The similarity of the TYSABRI®-associated adverse event profile between Study 1 (without co-administered AVONEX®) and Study 2 (with co-administered AVONEX®) indicates that this alteration in clearance does not necessitate reduction of the TYSABRI® dose to maintain safety **(see ADVERSE REACTIONS, General)**.

Results of studies in multiple sclerosis patients taking TYSABRI® and concomitant interferon beta-1a (AVONEX® 30 mcg IM once weekly) or glatiramer acetate were inconclusive with regard to the need for dose adjustment of the beta-interferon or glatiramer acetate.

Carcinogenesis, Mutagenesis, and Impairment of Fertility

No clastogenic or mutagenic effects of natalizumab were observed in the Ames or human chromosomal aberration assays. Natalizumab showed no effects on *in vitro* assays of α4-integrin positive tumor line proliferation/cytotoxicity. Xenograft transplantation models in SCID and nude

mice with two α4-integrin positive tumor lines (leukemia, melanoma) demonstrated no increase in tumor growth rates or metastasis resulting from natalizumab treatment.

Reductions in female guinea pig fertility were observed in one study at dose levels of 30 mg/kg, but not at the 10 mg/kg dose level (2.3-fold the clinical dose). A 47% reduction in pregnancy rate was observed in guinea pigs receiving 30 mg/kg relative to control. Implantations were seen in only 36% of animals having corpora lutea in the 30 mg/kg group versus 66-72% in the other groups. Natalizumab did not affect male fertility at doses up to 7-fold the clinical dose.

Pregnancy (Category C)

In reproductive studies in monkeys and guinea pigs, there was no evidence of teratogenic effects at doses up to 30 mg/kg (7 times the human clinical dose based on a body weight comparison). In one study where female guinea pigs were exposed to natalizumab during the second half of pregnancy, a small reduction in pup survival was noted at post-natal day 14 with respect to control (3 pups/litter for the group treated with 30 mg/kg natalizumab and 4.3 pups/litter for the control group). In one of five studies that exposed monkeys or guinea pigs during pregnancy, the number of abortions in treated (30 mg/kg) monkeys was 33% versus 17% in controls. No effects on abortion rates were noted in any other study. TYSABRI® underwent trans-placental transfer and produced *in utero* exposure in developing guinea pigs and cynomolgus monkeys. When pregnant dams were exposed to natalizumab at approximately 7-fold the clinical dose, serum levels in fetal animals at delivery were approximately 35% of maternal serum natalizumab levels. A study in pregnant cynomolgus monkeys treated at 2.3-fold the clinical dose demonstrated natalizumab-related changes in the fetus. These changes included mild anemia, reduced platelet count, increased spleen weights, and reduced liver and thymus weights associated with increased splenic extramedullary hematopoiesis, thymic atrophy, and decreased hepatic hematopoiesis. In offspring born to mothers treated with natalizumab at 7-fold the clinical dose, platelet counts were also reduced. This effect was reversed upon clearance of natalizumab. There was no evidence of anemia in these offspring.

There are no adequate and well-controlled studies of TYSABRI® therapy in pregnant women. Because animal reproduction studies are not always predictive of human response, this drug should be used during pregnancy only if clearly needed. If a woman becomes pregnant while taking TYSABRI®, discontinuation of TYSABRI® should be considered.

Nursing Mothers

It is not known whether TYSABRI® is excreted in human milk. Because many drugs and immunoglobulins are excreted in human milk, and because the potential for serious adverse reactions is unknown, a decision should be made whether to discontinue nursing or TYSABRI® taking into account the importance of therapy to the mother.

Geriatric Use

Clinical studies of TYSABRI® did not include sufficient numbers of patients aged 65 years and over to determine whether they respond differently than younger patients.

within 8 hours. If stored at 2-8°C, allow the solution to warm to room temperature prior to infusion. DO NOT FREEZE.

Administration Instructions

Infuse TYSABRI® 300 mg in 100 mL 0.9% Sodium Chloride Injection, USP over approximately one hour. After the infusion is complete, flush with 0.9% Sodium Chloride Injection, USP.

Use of filtration devices during administration has not been evaluated. Other medications should not be injected into infusion set side ports or mixed with TYSABRI®.

HOW SUPPLIED

TYSABRI® concentrate is supplied as 300 mg natalizumab in a sterile, single-use vial free of preservatives. Each package contains a single-use vial. NDC 59075-730-15

Storage

TYSABRI® single-use vials must be refrigerated between 2-8°C (36°-46°F). Do not use beyond the expiration date stamped on the carton and vial label. DO NOT SHAKE OR FREEZE. Protect from light.

If not used immediately, store the TYSABRI® solution for infusion at 2-8°C (36°-46°F). TYSABRI® solution for infusion must be administered within 8 hours of preparation.

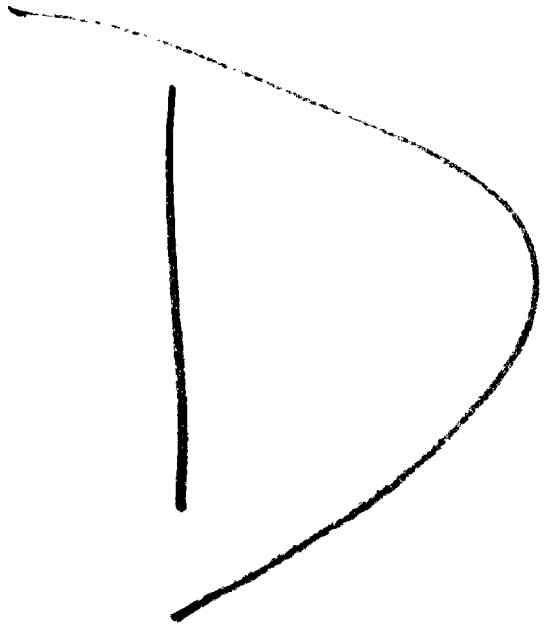
I61061-1 Issue date [November/2004]

TYSABRI® (natalizumab)

Manufactured by:
Biogen Idec Inc.
14 Cambridge Center
Cambridge, MA 02142 USA
1-888-489-7227

Distributed by:
Elan Pharmaceuticals, Inc.
San Diego, CA 92121

U.S. Patent Numbers: 5,840,299, 6,033,665, 6,602,503, 5,168,062, 5,385,839, 5,730,978



Attachment D

US005840299A

United States Patent [19]

Bendig et al.

[11] **Patent Number: 5,840,299**

[45] **Date of Patent: Nov. 24, 1998**

[54] **HUMANIZED ANTIBODIES AGAINST LEUKOCYTE ADHESION MOLECULE VLA-4**

[75] Inventors: **Mary M. Bendig**, London; **Olivier J. Léger**, Hertfordshire; **José Saldanha**, Enfield Middlesex; **S. Tarran Jones**, Radlett, all of United Kingdom; **Ted A. Yednock**, Fairfax, Calif.

[73] Assignee: **Athena Neurosciences, Inc.**, South San Francisco, Calif.

[21] Appl. No.: **561,521**

[22] Filed: **Nov. 21, 1995**

Related U.S. Application Data

[63] Continuation-in-part of Ser. No. 186,269, which is a continuation-in-part of PCT/US95/01219 Jan. 25, 1995, abandoned.

[51] **Int. Cl.**[6] **A61K 39/395**; C07K 16/28; C12P 21/08; C12N 15/13

[52] **U.S. Cl.** **424/133.1**; 424/130.1; 424/141.1; 424/143.1; 424/144.1; 424/153.1; 424/154.1; 424/173.1; 435/7.1; 435/7.2; 435/7.21; 435/7.24; 435/69.6; 435/172.3; 435/251.3; 435/320.1; 530/387.3; 530/388.73; 530/388.75; 530/388.22; 536/23.53

[58] **Field of Search** 424/130.1, 133.1, 424/141.1, 143.1, 144.1, 153.1, 154.1, 173.1; 435/69.6, 172.3, 252.3, 320.1, 7.1, 7.2, 7.21, 7.24; 536/23.4, 23.5, 23.53; 530/387.1, 387.3, 388.2, 388.22, 388.7, 388.73, 388.75

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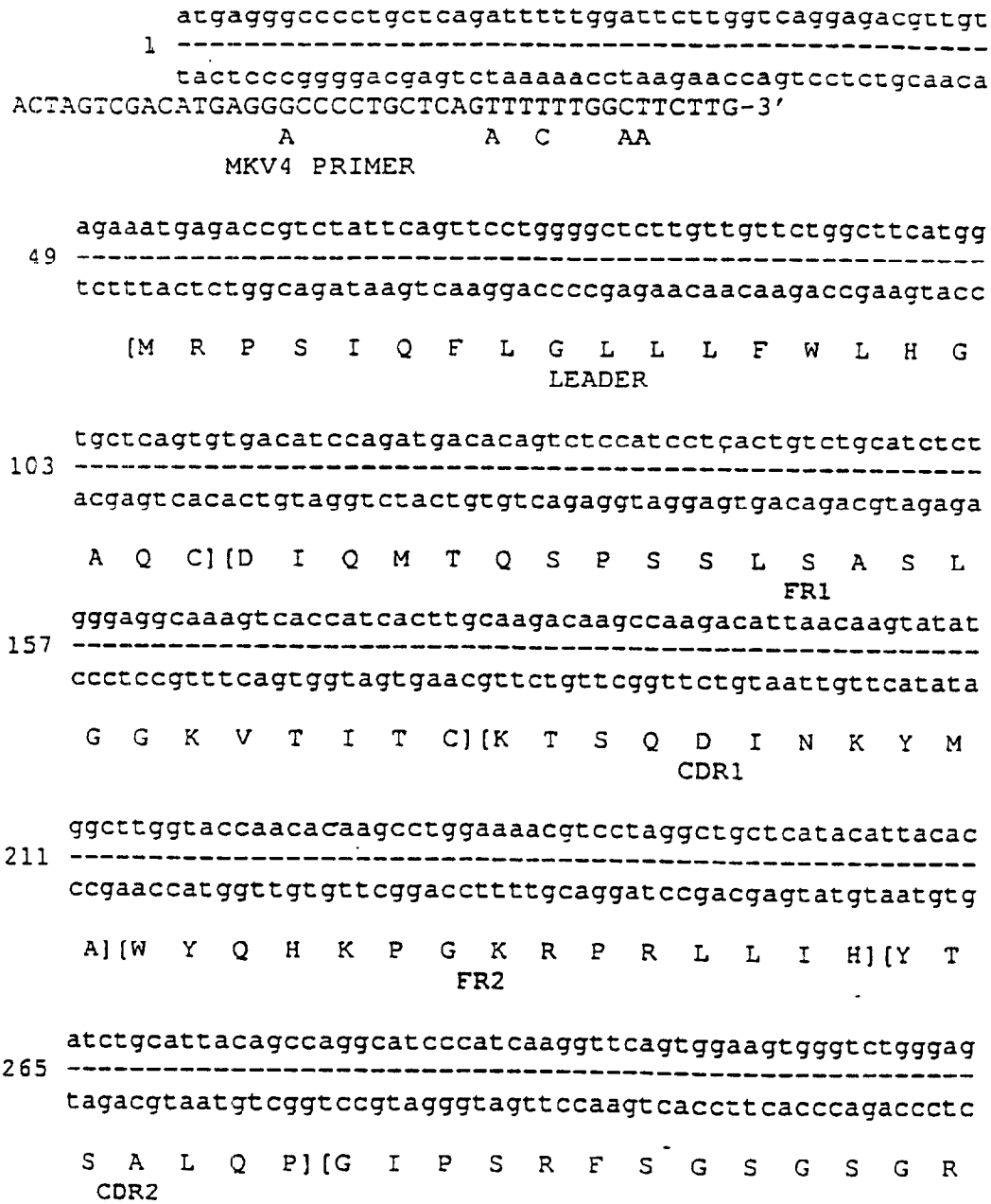
Primary Examiner—Lila Feisee
Assistant Examiner—Phillip Gambel
Attorney, Agent, or Firm—Townsend & Townsend & Crew LLP

[57] **ABSTRACT**

The invention provides humanized immunoglobulins that specifically bind to the VLA-4 ligand, and methods of treatment using the same. The methods are particularly useful for treatment of multiple sclerosis.

29 Claims, 16 Drawing Sheets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1
tactccccggggacgagtctaaaaacctaagaaccagtcctctgcaaca
ACTAGTCGACATGAGGGCCCCTGCTCAGTTTTTTGGCTTCTTG-3'
A A C AA
MKV4 PRIMER
agaaatgagaccgtctattcagttcctggggctcttgttgttctggcttcatgg
49
tctttactctggcagataagtcaaggaccccgagaacaacaagaccgaagtacc
[M R P S I Q F L G L L L F W L H G
LEADER
tgctcagtgtgacatccagatgacacagtctccatcctcactgtctgcatctct
103
acgagtcacactgtaggtctactgtgtcagaggtaggagtgacagacgtagaga
A Q C][D I Q M T Q S P S S L S A S L
FR1
gggaggcaaagtcaccatcacttgcaagacaagccaagacattaacaagtatat
157
ccctccgtttcagtggtagtgaacgttctgttcggttctgtaattgttcatata
G G K V T I T C][K T S Q D I N K Y M
CDR1
ggcttggtaccaacacaagcctggaaaacgtcctaggctgctcatacattacac
211
ccgaaccatggttgtgttcggacctttgcaggatccgacgagtatgtaatgtg
A][W Y Q H K P G K R P R L L I H][Y T
FR2
atctgcattacagccaggcatcccatcaaggttcagtggaagtgggtctgggag
265
tagacgtaatgtcggtccgtagggtagttccaagtcaccttcacccagaccctc
S A L Q P][G I P S R F S G S G S G R
CDR2

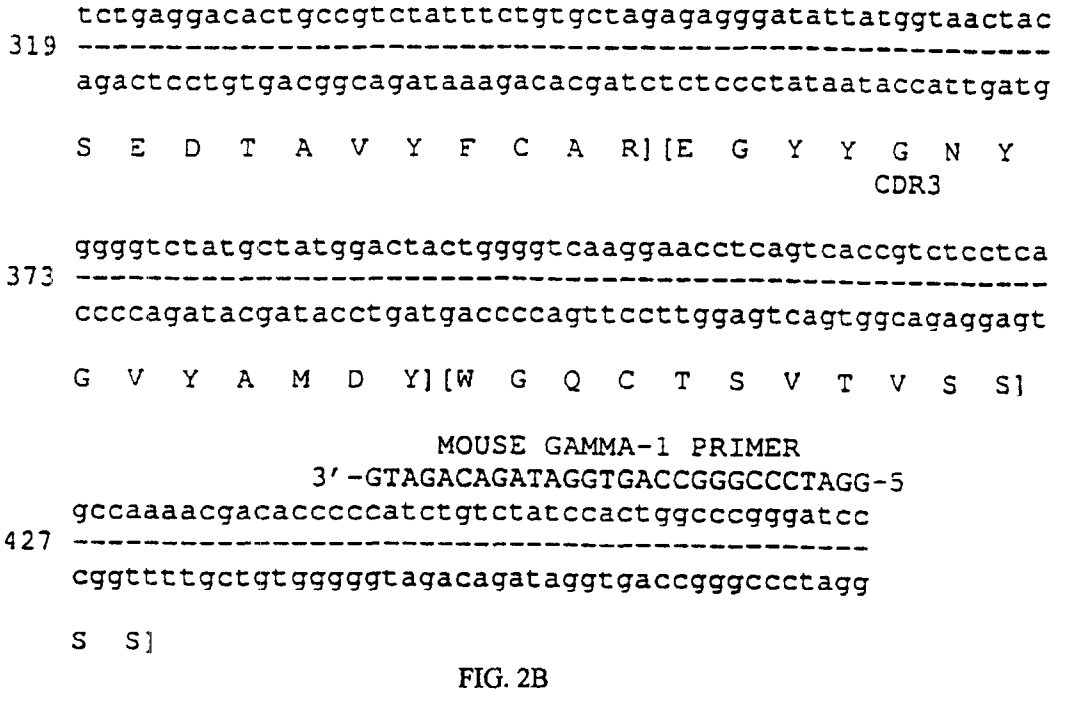
FIG. 1A

```
    agattattccttcaacatcagcaacctggagcctgaagatattgcaacttatta
319 ----------------------------------------------------
    tctaataaggaagttgtagtcgttggacctcggacttctataacgttgaataat

     D  Y  S  F  N  I  S  N  L  E  P  E  D  I  A  T  Y  Y
           FR3

    ttgtctacagtatgataatctgtggacgttcggtggaggcaccaagctggaaat
373 ----------------------------------------------------
    aacagatgtcatactattagacacctgcaagccacctccgtggttcgaccttta

     C][L  Q  Y  D  N  L  W  T][F  G  G  G  T  K  L  E  I
              CDR3                       FR4

                                      MOUSE KAPPA PRIMER
                                  3'-GTAGAAGGGTGGTAGGTGGGCCCT
    caaacgggctgatgctgcaccaactgtatccatcttcccaccatccacccggga
427 ----------------------------------------------------
    gtttgcccgactacgacgtggttgacataggtagaagggtggtaggtgggccct

     K]

    AGG-5'
    tcc
481 ---
    agg
```

FIG. 1B

```
          atgaaatgcagctgggtcatgttcttcctgatggcagtggttacaggg
        1 ----------------------------------------------
          tactttacgtcgacccagtacaagaaggactaccgtcaccaatgtccc
ACTAGTCGACATGAAATGCAGCTGGGTCATCTTCTTC-3'
                              G
          MHV1 PRIMER
         [M  K  C  S  W  V  M  F  F  L  M  A  V  V  T  G
                               LEADER

   gtcaattcagaggttcagctgcagcagtctggggcagagcttgtgaagccaggg
 49 ----------------------------------------------------
   cagttaagtctccaagtcgacgtcgtcagaccccgtctcgaacacttcggtccc

   V  N  S][E  V  Q  L  Q  Q  S  G  A  E  L  V  K  P  G
                                                FR1

   gcctcagtcaagttgtcctgcacagcttctggcttcaacattaaagacacctat
103 ----------------------------------------------------
   cggagtcagttcaacaggacgtgtcgaagaccgaagttgtaatttctgtggata

   A  S  V  K  L  S  C  T  A  S  G  F  N  I  K][D  T  Y
                                                   CDR1

   atacactgtgtgaagcagaggcctgaacagggcctggagtggattggaaggatt
157 ----------------------------------------------------
   tatgtgacacacttcgtctccggacttgtcccggacctcacctaaccttcctaa

   I  H][C  V  K  Q  R  P  E  Q  G  L  E  W  I  G][R  I
                               FR2

   gatcctgcgaatggttatactaaatatgacccgaagttccagggcaaggccact
211 ----------------------------------------------------
   ctaggacgcttaccaatatgatttatactgggcttcaaggtcccgttccggtga

   D  P  A  N  G  Y  T  K  Y  D  P  K  F  Q  G][K  A  T
                  CDR2

   ataacagctgacacatcctccaacacagcctacctgcagctcagcagcctgaca
265 ----------------------------------------------------
   tattgtcgactgtgtaggaggttgtgtcggatggacgtcgagtcgtcggactgt

   I  T  A  D  T  S  S  N  T  A  Y  L  Q  L  S  S  L  T
                                                FR3
```

FIG. 2A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319
agactcctgtgacggcagataaagacacgatctctccctataataccattgatg
S E D T A V Y F C A R][E G Y Y G N Y
CDR3
ggggtctatgctatggactactggggtcaaggaacctcagtcaccgtctcctca
373
ccccagatacgatacctgatgaccccagttccttggagtcagtggcagaggagt
G V Y A M D Y][W G Q C T S V T V S S]
MOUSE GAMMA-1 PRIMER
3'-GTAGACAGATAGGTGACCGGGCCCTAGG-5
gccaaaacgacacccccatctgtctatccactggcccgggatcc
427
cggttttgctgtgggggtagacagataggtgaccgggccctagg
S S]

FIG. 2B

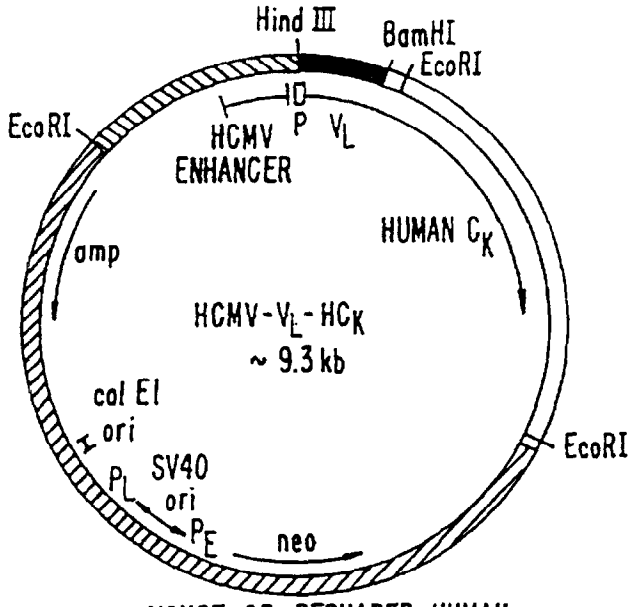

Hind III
BamHI
EcoRI
EcoRI
HCMV P V_L
ENHANCER
HUMAN C_K
amp
HCMV-V_L-HC_K
~ 9.3 kb
col EI
ori
SV40
ori
P_L
P_E
neo
EcoRI
■ V_L, MOUSE OR RESHAPED HUMAN
□ C_K, HUMAN
▨ HCMV
▨ pSV2neo

FIG. 3A.

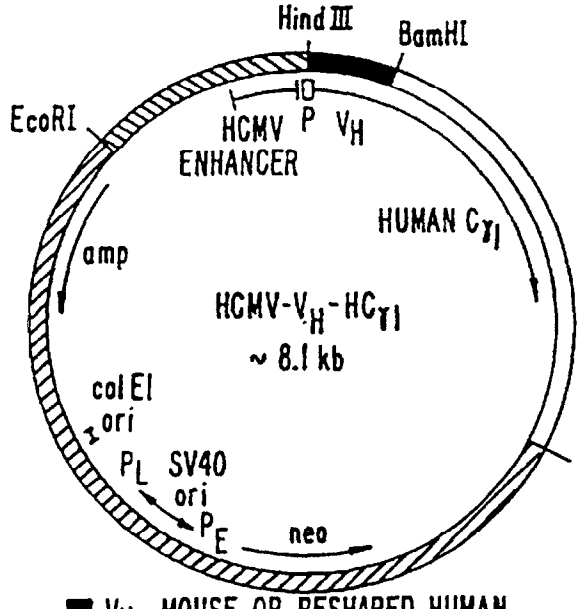

Hind III
BamHI
EcoRI
HCMV P V_H
ENHANCER
HUMAN $C_{\gamma 1}$
amp
HCMV-V_H-$HC_{\gamma 1}$
~ 8.1 kb
col EI
ori
P_L SV40
ori
P_E
neo
■ V_H, MOUSE OR RESHAPED HUMAN
□ $C_{\gamma 1}$, HUMAN
▨ HCMV
▨ pSV2neo

FIG. 3B.

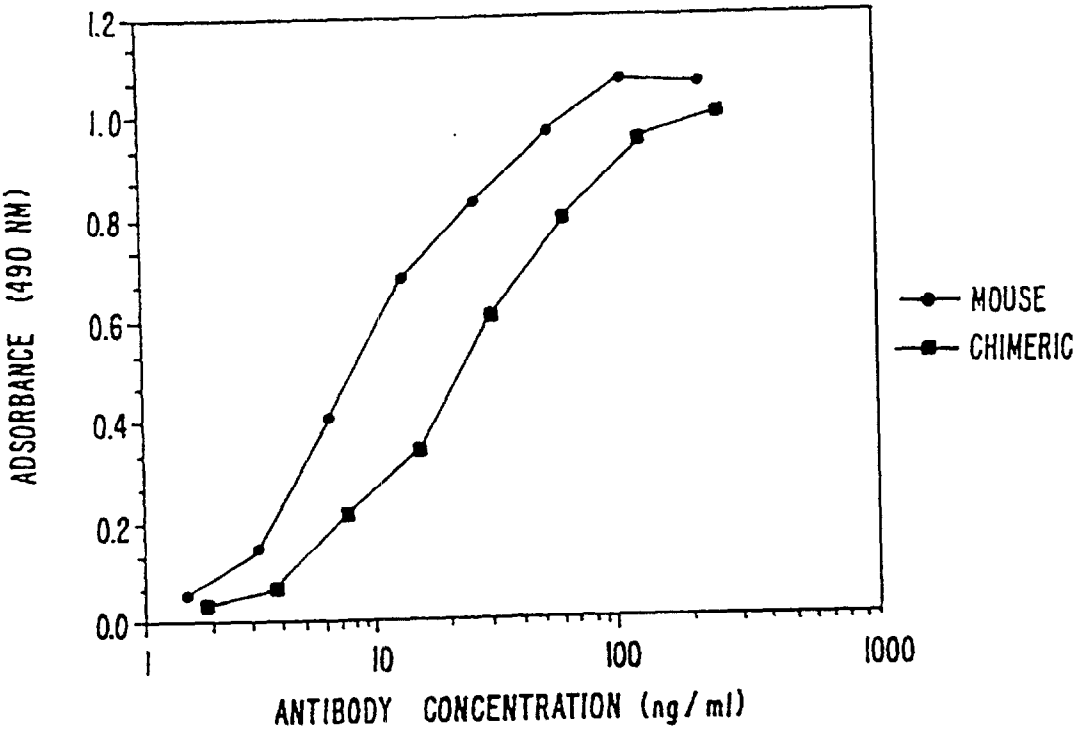

1.2
1.0
0.8
0.6
0.4
0.2
0.0
ADSORBANCE (490 NM)
1
10
100
1000
ANTIBODY CONCENTRATION (ng/ml)
MOUSE
CHIMERIC

FIG. 4.

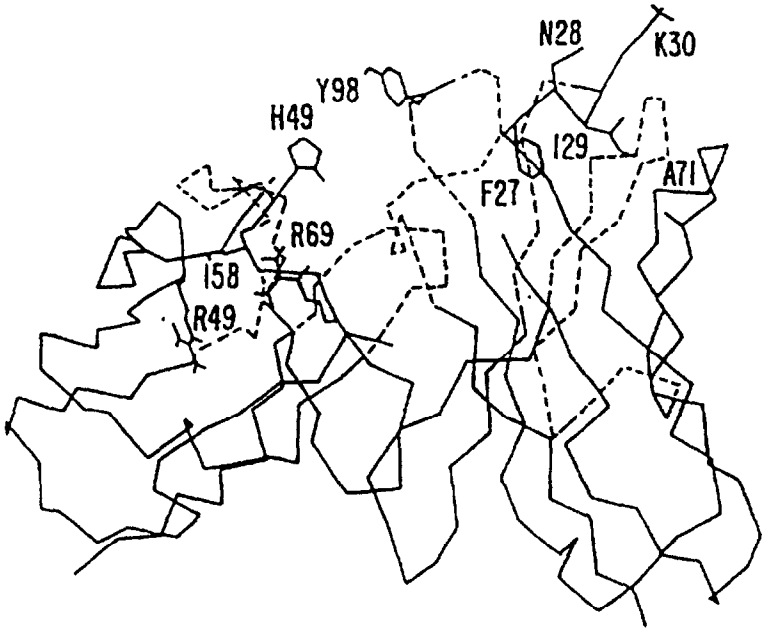

N28
K30
Y98
H49
I29
A71
F27
R69
I58
R49

FIG. 5.

```
               FR1                     CDR1        FR2              CDR2
                1           2            3           4               5
     12345678901234567890123 45678901234 567890123456789 0123456
      *                        *********               * ***
21.6 DIQMTQSPSSLSASLGGKVTITC KTSQDINKYMA WYQHKPGKRPRLLIH YTSALQP

REI  DIQMTQSPSSLSASVGDRVTITC QASQDIIKYLN WYQQTPGKAPKLLIY EASNLQA

La   DIQMTQSPSSLSASVGDRVTITC KTSQDINKYMA WYQQTPGKAPRLLIH YTSALQP

Lb   ----------------------- ----------- ----------R---- -------


                 FR3                        CDR3     FR4
      6         7         8         9         10
     7890123456789012345678901234567890 901234567 8901234567
            *        *                   *******
21.6 GIPSRFSGSGSGRDYSFNISNLEPEDIATYYC LQYDNL-WT FGGGTKLEIK

REI  GVPSRFSGSGSGTDYTFTISSLQPEDIATYYC QQYQSLPYT FGQGTKLQIT

La   GIPSRFSGSGSGRDYTFTISSLQPEDIATYYC LQYDNL-WT FGQGTKVEIK

Lb   -I----------R------------------- --------- ------VE-K
```

FIG. 6.

```
                       FR1                      CDR1       FR2           CDR2
                   1         2         3               4             5         6
     123456789012345678901234567890 12345 67890123456789 012A3456789012345
                               ***** ** *                   ****
21.6 EVQLQQSGAELVKPGASVKLSCTASGFNIK DTYIH CVKQRPEQGLEWIG RIDPANGYTKYDPKFQG

2*CL QVQLVQSGAEVKKPGASVKVSCKASGYTFT SYAMH WVRQAPGQRLEWMG WINAGNGNTKYSQKFQG

Ha   QVQLVQSGAEVKKPGASVKVSCKASGFNIK DTYIH WVRQAPGQRLEWMB RIDPANGYTKYDPKFQG

Hb   --------------------------FNIK ----- --------G----- -----------------

Hc   --------------------------FNIK ----- -------------- -----------------


                  FR3                    CDR3        FR4
      7         8            9         10              11
     67890123456789012ABC345678901234 567890ABCDEF12 34567890123
          *                         *
21.6 KATITADTSSNTAYLQLSSLTSEDTAVYFCAR EGYYGNYGVYAMDY WGQGTSVTVSS

2*CL RVTITRDTSASTAYMELSSLRSEDTAVYYCAR GGYYGSGS----NY WGQGTLVTVSS

Ha   RVTITADTSASTAYMELSSLRSEDTAVYYCAR EGYYGNYGVYAMDY WGQGTLVTVSS

Hb   -----A-------------------------- -------------- -----------

Hc   -----A-------------------------- ---F---------- -----------
```

FIG. 7.

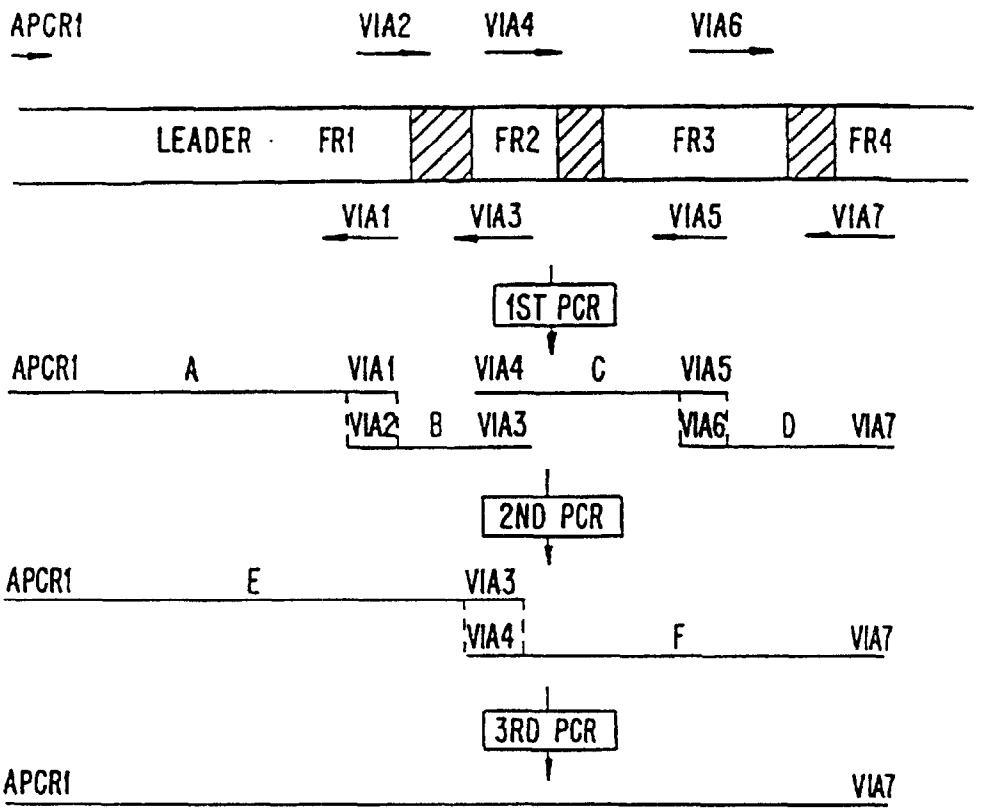

APCR1
VIA2
VIA4
VIA6
LEADER
FR1
FR2
FR3
FR4
VIA1
VIA3
VIA5
VIA7
1ST PCR
APCR1
A
VIA1
VIA4
C
VIA5
VIA2
B
VIA3
VIA6
D
VIA7
2ND PCR
APCR1
E
VIA3
VIA4
F
VIA7
3RD PCR
APCR1
VIA7

FIG. 8.

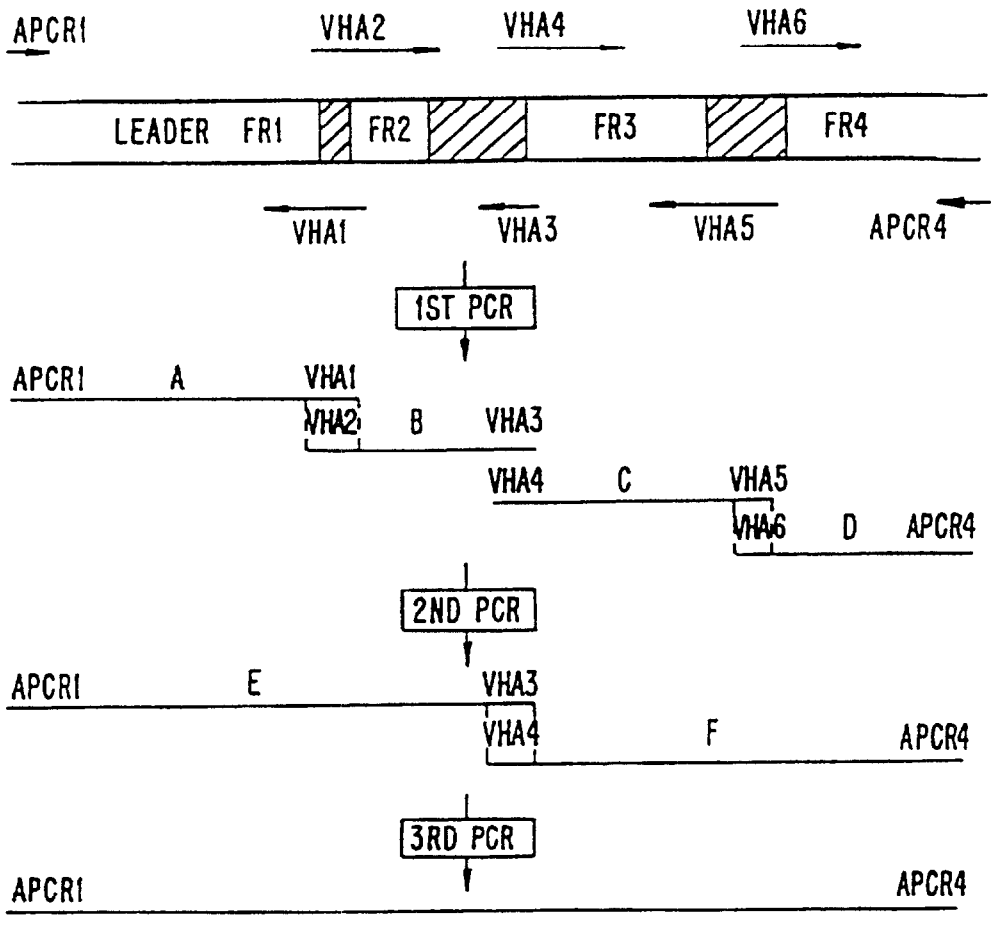
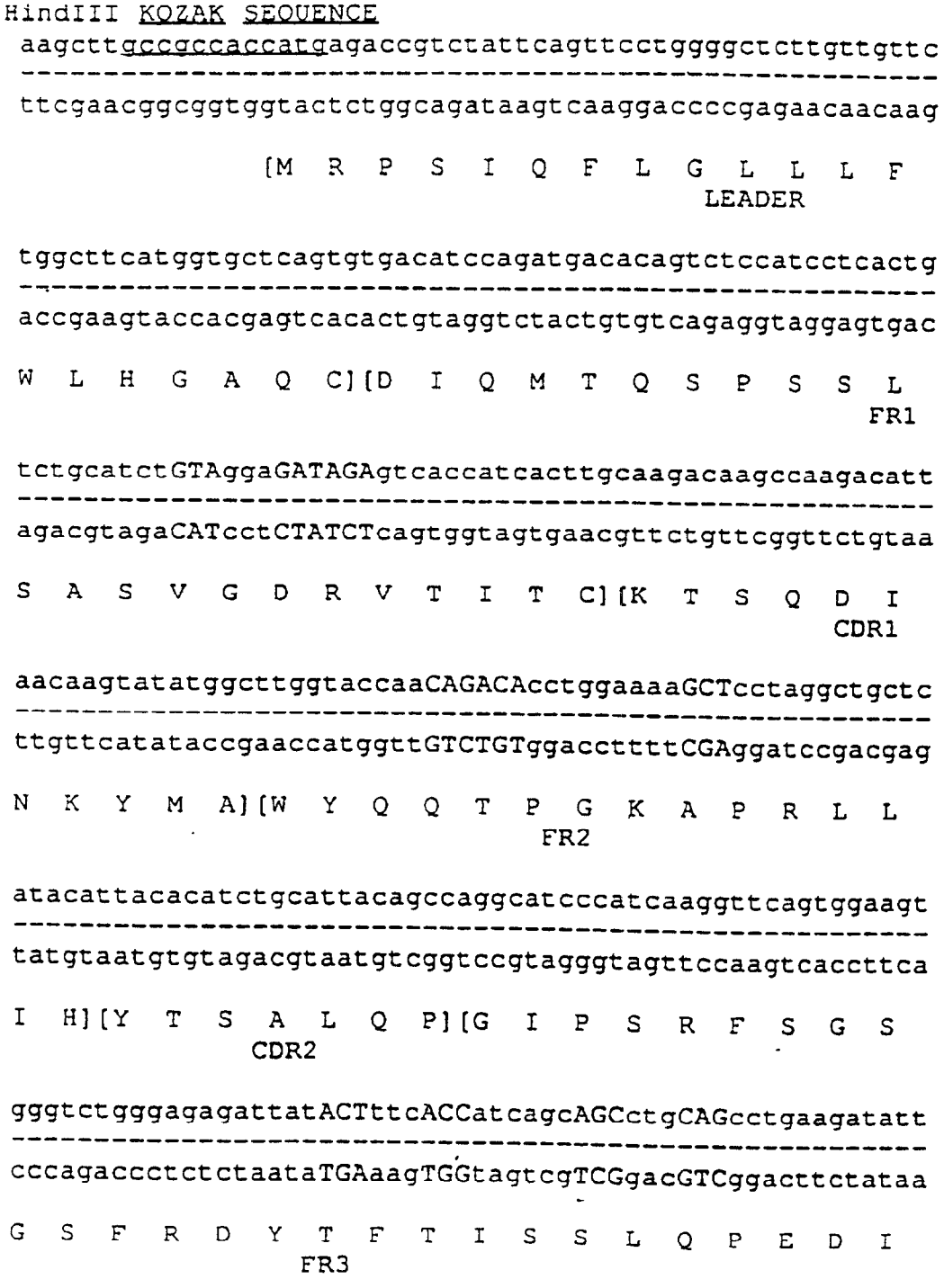

APCR1
VHA2
VHA4
VHA6
LEADER
FR1
FR2
FR3
FR4
VHA1
VHA3
VHA5
APCR4
1ST PCR
APCR1
A
VHA1
VHA2
B
VHA3
VHA4
C
VHA5
VHA6
D
APCR4
2ND PCR
APCR1
E
VHA3
VHA4
F
APCR4
3RD PCR
APCR1
APCR4

FIG. 9.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1
ttcgaacggcggtggtactctggcagataagtcaaggaccccgagaacaacaag
[M R P S I Q F L G L L L F
LEADER
tggcttcatggtgctcagtgtgacatccagatgacacagtctccatcctcactg
55
accgaagtaccacgagtcacactgtaggtctactgtgtcagaggtaggagtgac
W L H G A Q C][D I Q M T Q S P S S L
FR1
tctgcatctGTAggaGATAGAgtcaccatcacttgcaagacaagccaagacatt
109
agacgtagaCATcctCTATCTcagtggtagtgaacgttctgttcggttctgtaa
S A S V G D R V T I T C][K T S Q D I
CDR1
aacaagtatatggcttggtaccaaCAGACAcctggaaaaGCTcctaggctgctc
163
ttgttcatataccgaaccatggttGTCTGTggacctttTCGAggatccgacgag
N K Y M A][W Y Q Q T P G K A P R L L
FR2
atacattacacatctgcattacagccaggcatcccatcaaggttcagtggaagt
217
tatgtaatgtgtagacgtaatgtcggtccgtagggtagttccaagtcaccttca
I H][Y T S A L Q P][G I P S R F S G S
CDR2
gggtctgggagagattatACTttcACCatcagcAGCctgCAGcctgaagatatt
271
cccagaccctctctaataTGAaagTGGtagtcgTCGgacGTCggacttctataa
G S F R D Y T F T I S S L Q P E D I
FR3

FIG. 10A

```
    gcaacttattattgtctacagtatgataatctgtggacgttcggtCAAggcacc
325 ----------------------------------------------------
    cgttgaataataacagatgtcatactattagacacctgcaagccaGTTccgtgg

    A  T  Y  Y  C][L  Q  Y  D  N  L  W  T][F  G  Q  G  T
                              CDR3                    FR4

        SPLICE DONOR SITE BamHI
    aagGTGgaaatcaaacgtgagtggatcc
379 ----------------------------
    ttcCACctttagtttgcactcacctagg

    K  V  E  I  K]
```

FIG. 10B

```
  HindIII KOZAK SEQUENCE
    AAGCTTGCCGCCACCATGGACTGGACCTGGCGCGTGTTTTGCCTGCTCGCCGTG
  1 ----------------------------------------------------
    TTCGAACGGCGGTGGTACCTGACCTGGACCGCGCACAAAACGGACGAGCGGCAC

                  [M  D  W  T  W  R  V  F  C  L  L  A  V
                                              LEADER

    GCTCCTGGGGCCCACAGCCAGGTGCAACTAGTGCAGTCCGGCGCCGAAGTGAAG
 55 ----------------------------------------------------
    CGAGGACCCCGGGTGTCGGTCCACGTTGATCACGTCAGGCCGCGGCTTCACTTC

    A  P  G  A  H  S][Q  V  Q  L  V  Q  S  G  A  E  V  K


    AAACCCGGTGCTTCCGTGAAAGTCAGCTGTAAAGCTAGCGGTttcaacattaaa
109 ----------------------------------------------------
    TTTGGGCCACGAAGGCACTTTCAGTCGACATTTCGATCGCCAaagttgtaattt

    K  P  G  A  S  V  K  V  S  C  K  A  S  G  F  N  I  K][
          FR1

    gacacctatatacacTGGGTTAGACAGGCCCCtGGCCAAaGGCTgGAGTGGATg
163 ----------------------------------------------------
    ctgtggatatatgtgACCCAATCTGTCCGGgGaCCGGTTtCCGAcCTCACCTAc

    D  T  Y  I  H][W  V  R  Q  A  P  G  Q  R  L  E  W  M
       CDR1                       FR2
```

FIG. 11A

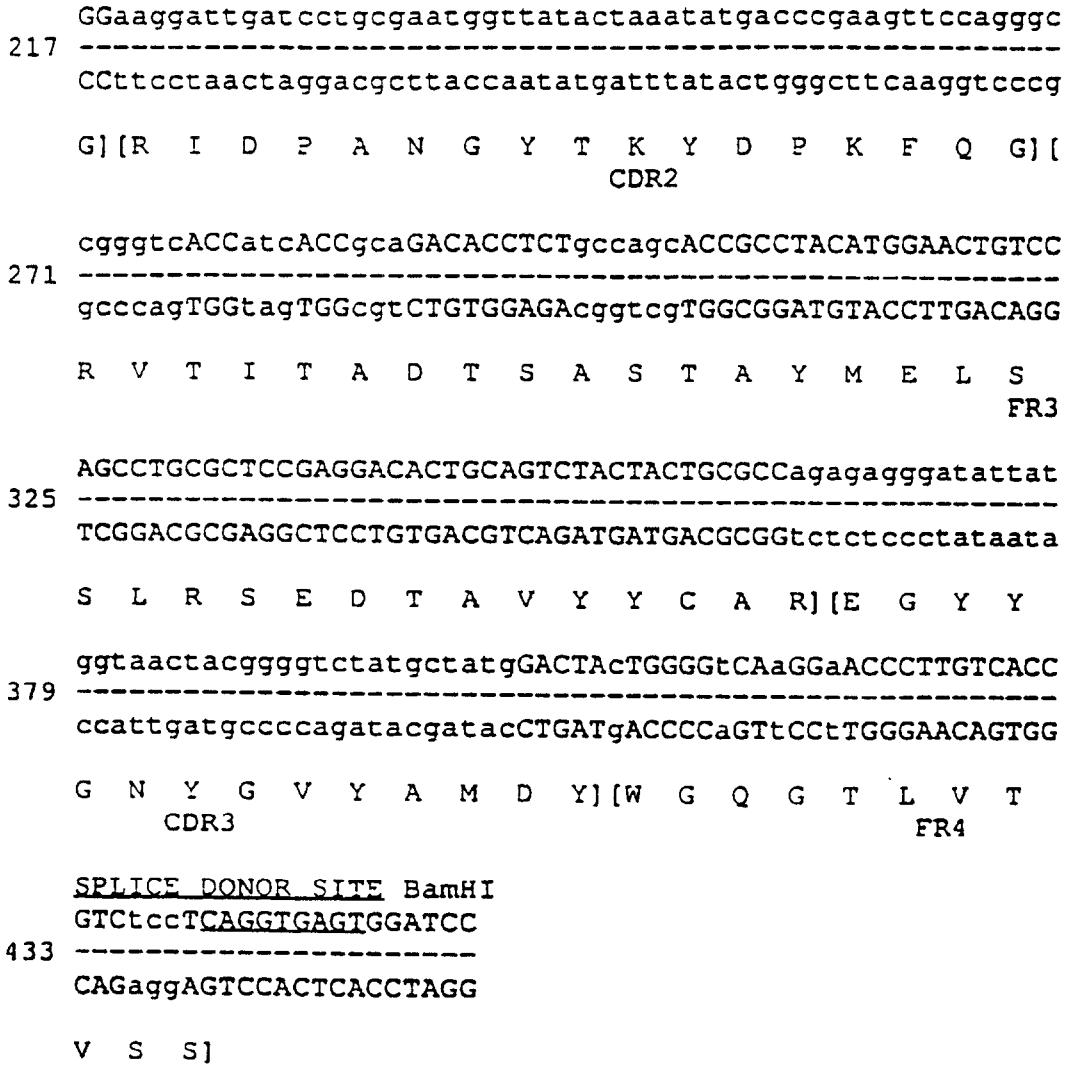
GGaaggattgatcctgcgaatggttatactaaatatgacccgaagttccagggc
217
CCttcctaactaggacgcttaccaatatgatttatactgggcttcaaggtcccg
G][R I D P A N G Y T K Y D P K F Q G][
CDR2
cgggtcACCatcACCgcaGACACCTCTgccagcACCGCCTACATGGAACTGTCC
271
gcccagTGGtagTGGcgtCTGTGGAGAcggtcgTGGCGGATGTACCTTGACAGG
R V T I T A D T S A S T A Y M E L S
FR3
AGCCTGCGCTCCGAGGACACTGCAGTCTACTACTGCGCCagagagggatattat
325
TCGGACGCGAGGCTCCTGTGACGTCAGATGATGACGCGGtctctccctataata
S L R S E D T A V Y Y C A R][E G Y Y
ggtaactacggggtctatgctatgGACTAcTGGGGtCAaGGaACCCTTGTCACC
379
ccattgatgccccagatacgatacCTGATgACCCCaGTtCCtTGGGAACAGTGG
G N Y G V Y A M D Y][W G Q G T L V T
CDR3
FR4
SPLICE DONOR SITE BamHI
GTCtccTCAGGTGAGTGGATCC
433
CAGaggAGTCCACTCACCTAGG
V S S]

FIG 11B

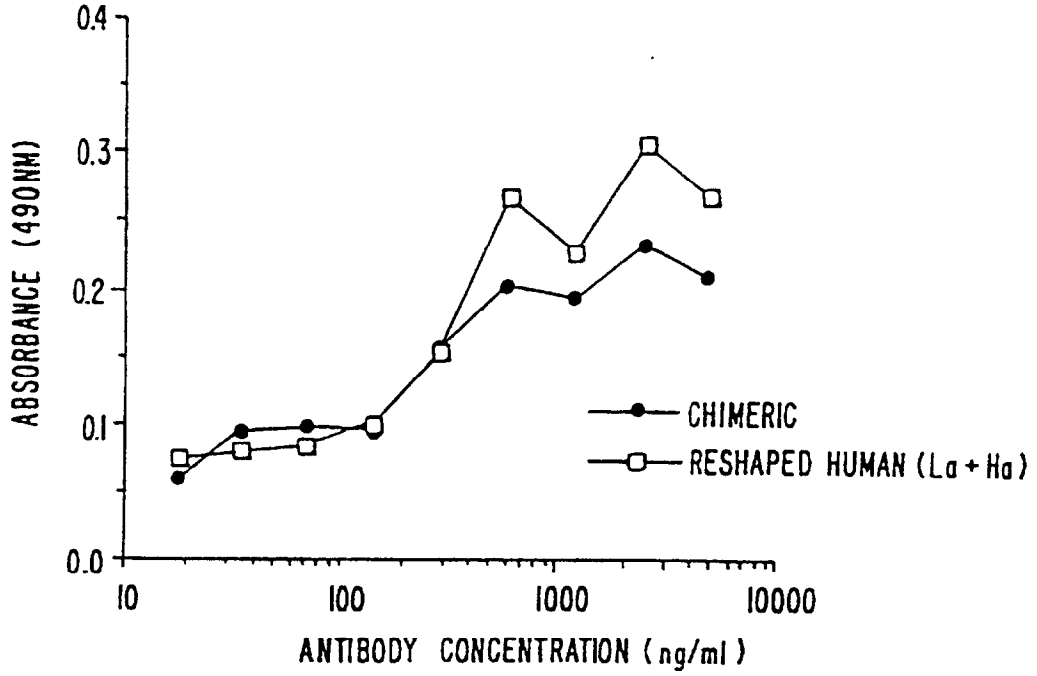

FIG. 12A.

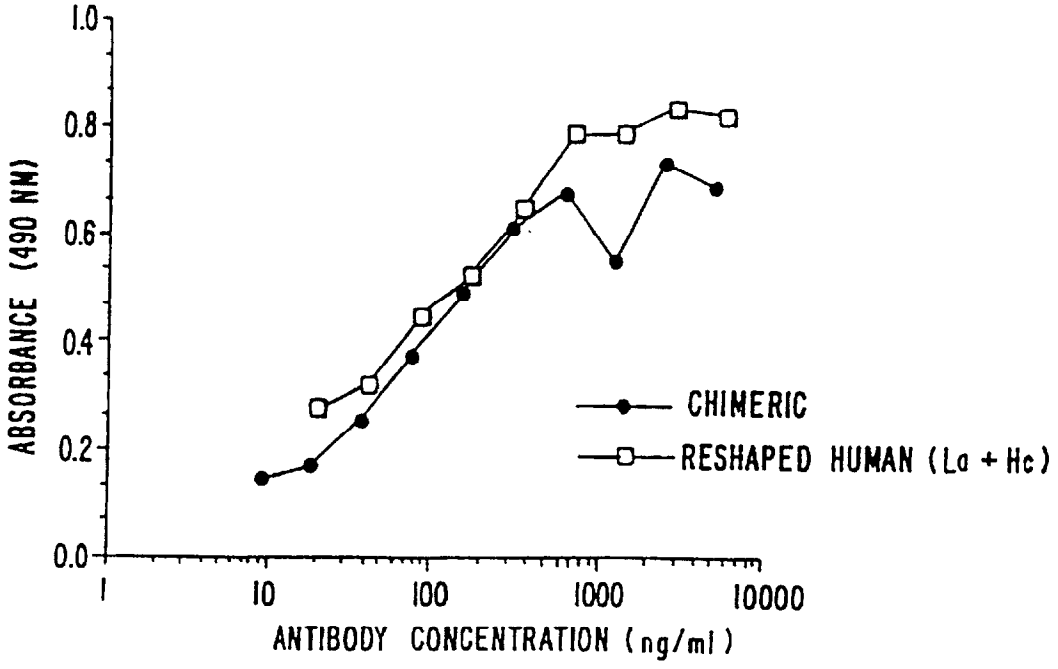

FIG. 12B.

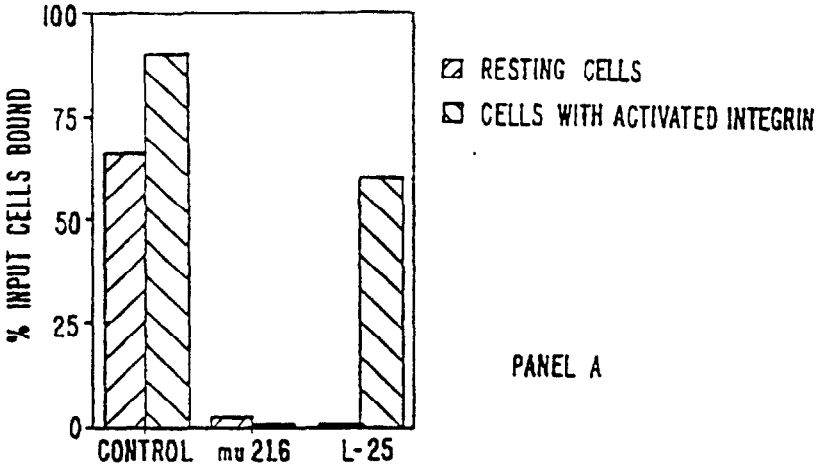

FIG. 13A.

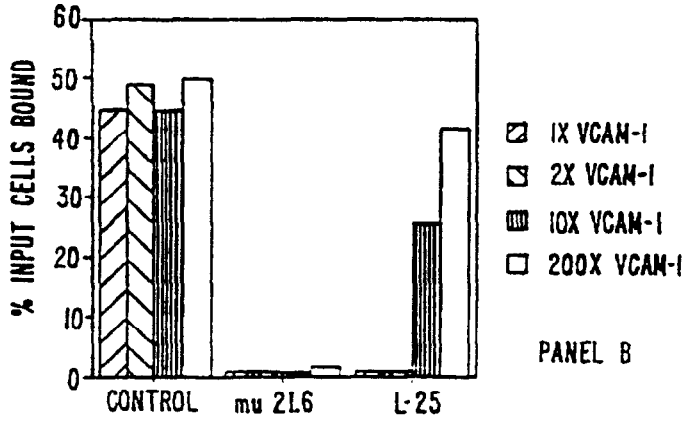

FIG. 13B.

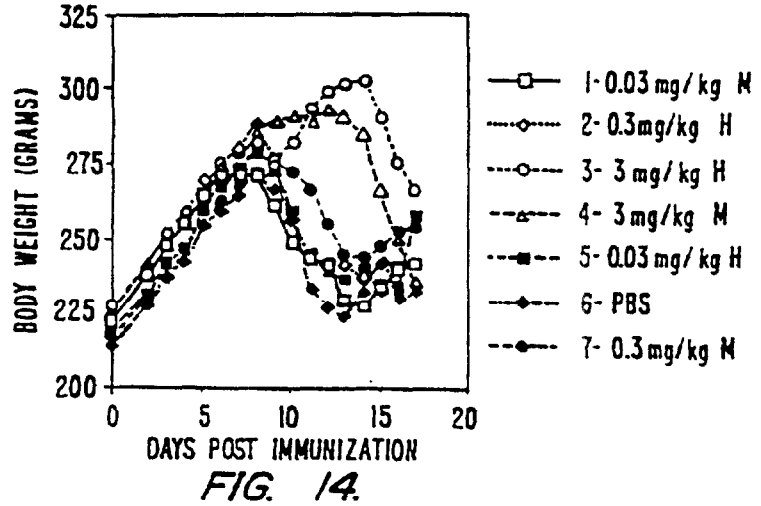

FIG. 14.

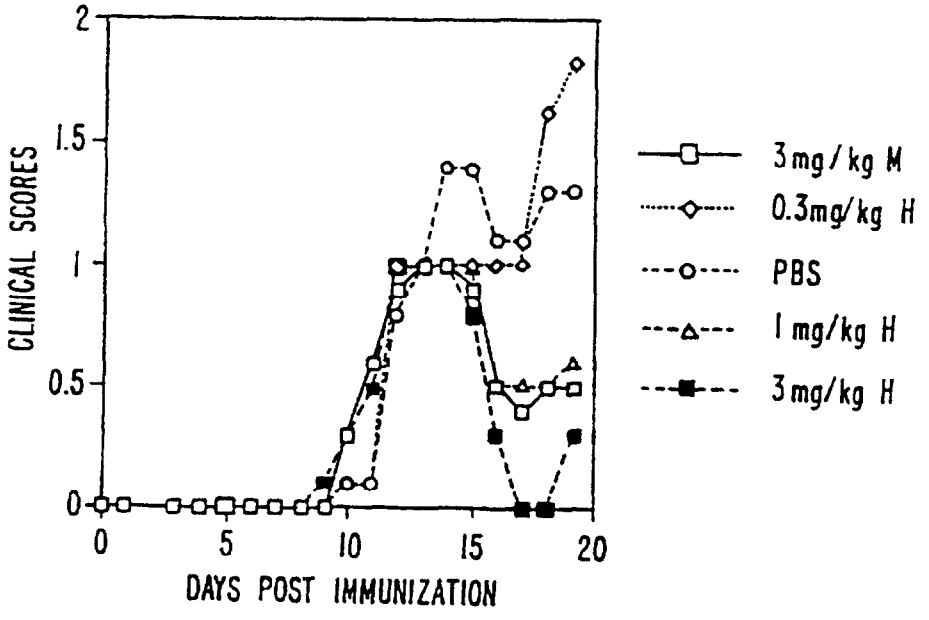
CLINICAL SCORES
2
1.5
1
0.5
0
0
5
10
15
20
DAYS POST IMMUNIZATION
3mg/kg M
0.3mg/kg H
PBS
1 mg/kg H
3mg/kg H

FIG. 15.

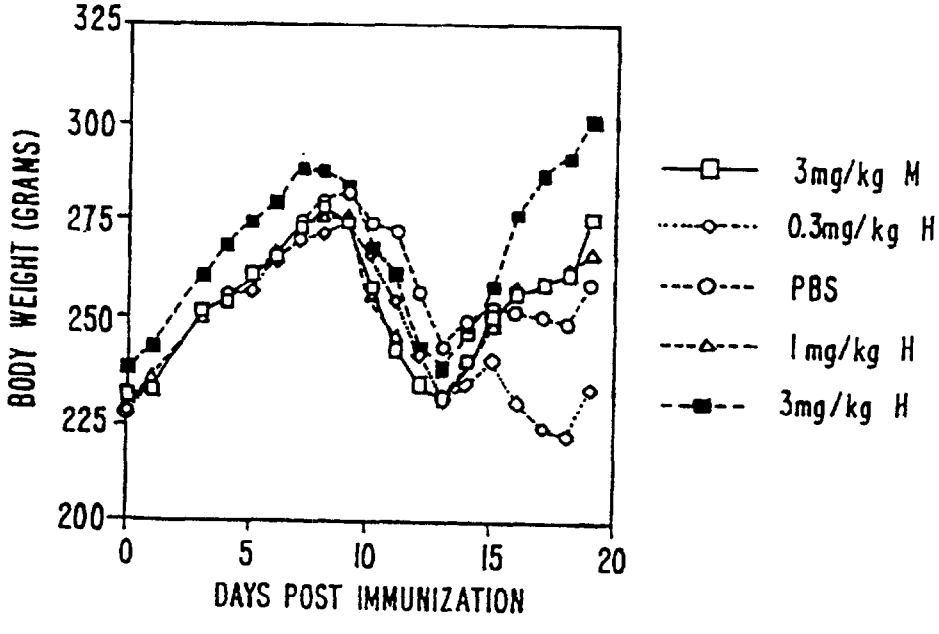
BODY WEIGHT (GRAMS)
325
300
275
250
225
200
0
5
10
15
20
DAYS POST IMMUNIZATION
3mg/kg M
0.3mg/kg H
PBS
1 mg/kg H
3mg/kg H

FIG. 16.

HUMANIZED ANTIBODIES AGAINST LEUKOCYTE ADHESION MOLECULE VLA-4

CROSSREFERENCE TO RELATED APPLICATIONS

This application is a continuation-in-part of PCT/US95/01219, filed Jan. 25, 1995, which is a continuation-in-part of U.S. Ser. No. 08/186,269, (now abandoned) filed Jan. 25, 1994, both of which are incorporated by reference in their entirety for all purposes.

TECHNICAL FIELD

This invention relates generally to humanized antibodies specific for the alpha-4 subunit of leukocyte adhesion molecule VLA-4.

BACKGROUND OF THE INVENTION

Inflammation is a response of vascularized tissues to infection or injury and is effected by adhesion of leukocytes to the endothelial cells of blood vessels and their infiltration into the surrounding tissues. In normal inflammation, the infiltrating leukocytes release toxic mediators to kill invading organisms, phagocytize debris and dead cells, and play a role in tissue repair and the immune response. However, in pathologic inflammation, infiltrating leukocytes are overresponsive and can cause serious or fatal damage. See, e.g., Hickey, *Psychoneuroimmunology II* (Academic Press 1990).

The attachment of leukocytes to endothelial cells is effected via specific interaction of cell-surface ligands and receptors on endothelial cells and leukocytes. See generally Springer, *Nature* 346:425–433 (1990). The identity of the ligands and receptors varies for different cell subtypes, anatomical locations and inflammatory stimuli. The VLA-4 leukocyte cell-surface receptor was first identified by Hemler, EP 330,506 (1989) (incorporated by reference in its entirety for all purposes). VLA-4 is a member of the β1 integrin family of cell surface receptors, each of which comprises α and β chains. VLA-4 contains an α4 chain and a β1 chain. VLA-4 specifically binds to an endothelial cell ligand termed VCAM-1. See Elices et al., *Cell* 60:577–584 (1990) (incorporated by reference in its entirety for all purposes). Although VCAM-1 was first detected on activated human umbilical vein cells, this ligand has also been detected on brain endothelial cells. See commonly owned, co-pending application U.S. Ser. No. 07/871,223 (incorporated by reference in its entirety for all purposes).

Adhesion molecules such as VLA-4, are potential targets for therapeutic agents. The VLA-4 receptor is a particularly important target because of its interaction with a ligand residing on brain endothelial cells. Diseases and conditions resulting from brain inflammation have particularly severe consequences. For example, one such disease, multiple sclerosis (MS), has a chronic course (with or without exacerbations and remissions) leading to severe disability and death. The disease affects an estimated 250,000 to 350,000 people in the United States alone.

Antibodies against the VLA-4 receptor have been tested for their anti-inflammatory potential both in vitro and in vivo in animal models. See U.S. Ser. No. 07/871,223 and Yednock et al, *Nature* 356:63–66 (1992) (incorporated by reference in its entirety for all purposes). The in vitro experiments demonstrate that anti-VLA-4 antibodies block attachment of lymphocytes to brain endothelial cells. The animal experiments test the effect of anti-VLA-4 antibodies on animals having an artificially induced condition (experimental autoimmune encephalomyelitis), simulating multiple sclerosis. The experiments show that administration of anti-VLA-4 antibodies prevents inflammation of the brain and subsequent paralysis in the animals. Collectively, these experiments identify anti-VLA-4 antibodies as potentially useful therapeutic agents for treating multiple sclerosis and other inflammatory diseases and disorders.

A significant problem with the anti-VLA-4 antibodies available to-date is that they are all of murine origin, and therefore likely to raise a human anti-mouse response (HAMA) in clinical use. A HAMA response reduces the efficacy of mouse antibodies in patients and prevents continued administration. One approach to this problem is to humanize mouse antibodies. In this approach, complementarity determining regions (CDRs) and certain other amino acids from donor mouse variable regions are grafted into human variable acceptor regions and then joined to human constant regions. See, e.g., Riechmann et al., *Nature* 332:323–327 (1988); Winter, U.S. Pat. No. 5,225,539 (1993) (each of which is incorporated by reference in its entirety for all purposes).

Although several examples of humanized antibodies have been produced, the transition from a murine to a humanized antibody involves a compromise of competing considerations, the solution of which varies with different antibodies. To minimize immunogenicity, the immunoglobulin should retain as much of the human acceptor sequence as possible. However, to retain authentic binding properties, the immunoglobulin framework should contain sufficient substitutions of the human acceptor sequence to ensure a three-dimensional conformation of CDR regions as close as possible to that in the original mouse donor immunoglobulin. As a result of these competing considerations, many humanized antibodies produced to-date show some loss of binding affinity compared with the corresponding murine antibodies from which they are derived. See, e.g., Jones et al., *Nature* 321:522–525 (1986); Shearman et al., *J. Immunol.* 147:4366–4373 (1991); Kettleborough et al., *Protein Engineering* 4:773–783 (1991); Gorman et al., *Proc. Natl. Acad. Sci. USA* 88:4181–4185 (1991); Tempest et al., *Biotechnology* 9:266–271 (1991).

Based on the foregoing it is apparent that a need exists for humanized anti-VLA-4 antibodies demonstrating a strong affinity for the VLA-4 receptor, while exhibiting little, if any, human-antimouse response. The present invention fulfill this and other needs.

SUMMARY OF THE INVENTION

The invention provides humanized immunoglobulins that specifically bind to a VLA-4 ligand. The humanized antibodies comprise a humanized light chain and a humanized heavy chain. The humanized light chain comprises three complementarity determining regions (CDR1, CDR2 and CDR3) having amino acid sequences from the corresponding complementarity determining regions of a mouse 21-6 immunoglobulin light chain, and a variable region framework from a human kappa light chain variable region framework sequence except in at least one position selected from a first group consisting of positions L45, L49, L58 and L69, wherein the amino acid position is occupied by the same amino acid present in the equivalent position of the mouse 21.6 immunoglobulin light chain variable region framework. The humanized heavy chain comprises three complementarity determining regions (CDR1, CDR2 and CDR3) having amino acid sequences from the correspond-

ing complementarity determining regions of a mouse 21-6 immunoglobulin heavy chain, and a variable region framework from a human heavy chain variable region framework sequence except in at least one position selected from a group consisting of H27, H28, H29, H30, H44, H71, wherein the amino acid position is occupied by the same amino acid present in the equivalent position of the mouse 21-6 immunoglobulin heavy. chain variable region framework The immunoglobulins specifically bind to VLA-4 with an affinity having a lower limit of about 10^7M^{-1} and an upper limit of about five times the affinity of the mouse 21-6 immunoglobulin.

Usually, the humanized light and heavy chain variable region frameworks are from REI and 21/28'CL variable region framework sequences respectively. When the humanized light chain variable region framework is from REI, at least two framework amino acids are replaced. One amino acid is from the first group of positions described supra. The other amino acids is from a third group consisting of positions L104, L105 and L107. This position is occupied by the same amino acid present in the equivalent position of a kappa light chain from a human immunoglobulin other than REI.

Some humanized immunoglobulins have a mature light chain variable region sequence designated La or Lb in FIG. 6, or a mature heavy chain variable region sequence designated Ha, Hb or Hc in FIG. 7. Preferred humanized immunoglobulins include those having an La light chain and an Ha, Hb or Hc heavy chain.

The invention also provides binding fragments of the humanized immunoglobulins against VLA-4 described supra.

In another aspect, the invention provides nucleic acids encoding the humanized immunoglobulins against VLA-4 described supra.

Also provided are computers programmed to display three dimensional images of the mouse 21.6 antibody or the humanized immunoglobulins described supra.

In another aspect the invention provides pharmaceutical compositions and methods of treatment using the same. The pharmaceutical compositions comprise a humanized immunoglobulin or binding fragment as described supra, and a pharmaceutically acceptable carrier. In some methods of treatment a therapeutically effective amount of a pharmaceutical composition is administered to a patient suffering from an inflammatory disease, such as multiple sclerosis.

Also provided are methods of detecting VLA-4 antigen using the humanized immunoglobulins and binding fragments described supra. In these methods, a humanized antibody or binding fragment is administered to a patient or a tissue sample therefrom. Complexes formed by specific binding between the antibody or fragment and VLA-4 present in the sample are detected.

BRIEF DESCRIPTION OF FIGURES

FIGS. 1A and 1B: DNA (SEQ. ID NO:1) and amino acid (SEQ. ID NO:2) sequences of the mouse 21.6 light chain variable region.

FIGS. 2A and 2B: DNA (SEQ. ID NO:3) and amino acid (SEQ. ID NO:4) sequences of the mouse 21.6 heavy chain variable region

FIG. 3: Light (A) and heavy (B) chain expression vectors used to produce chimeric and reshaped human antibodies with human kappa light chains and human gamma-1 heavy chains in mammalian cells.

FIG. 4: ELISA comparison of chimeric and mouse 21.6 antibody binding to L cells expressing human $\alpha4\beta1$ integrin on their surface.

FIG. 5: Molecular model of the variable regions of mouse 21.6 antibody. Residues of special interest are labelled.

FIG. 6: Comparisons of the amino acid sequences of mouse and reshaped human 21.6 (SEQ. ID NO:5) light chain variable regions. The amino acid residues that are part of the Chothia canonical sequences for the CDR loop structures are marked with an asterisk. REI (SEQ. ID NO:6) shows the FRs and CDRs from the V_L region of human REI light chain. La (SEQ. ID NO:7) and Lb (SEQ. ID NO:8) are the two versions of reshaped human 21.6 V_L region. The residues in the FRs of La that differ from those in the REI sequence are underlined. In Lb, only the residues in the framework regions that differ from those of REI are shown.

FIG. 7: Comparisons of the amino acid sequences of the mouse and reshaped human 21.6 (SEQ. ID NO:9) heavy chain variable regions. The amino acid residues that are part of the canonical sequences for the Chothia CDR loop structures are marked with an asterisk. 2*CL (SEQ. ID NO:10) shows the FRs and CDRs from the V_H region of human 21/28'CL antibody. Ha (SEQ. ID NO:11), Hb (SEQ. ID NO:12), and Hc (SEQ. ID NO:13) are the three versions of reshaped human 21.6 V_H region. The residues in the FRs of Ha that differ from those in the 21/28'CL sequence are underlined. In Hb and Hc, only the residues in the framework regions that differ from those of 21/28'CL are shown.

FIG. 8: PCR-based construction of version "a" of reshaped human 21.6 light chain variable region. The dotted lines indicate a complementary sequence of at least 21 bases between the primers.

FIG. 9: PCR-based construction of version "a" of reshaped human 21.6 heavy chain variable region.

FIGS. 10A and 10B: cDNA and amino acid sequences (SEQ. ID NOS. 14 and 15) of the first version ("a") of reshaped human 21.6 light chain variable region.

FIGS. 11A and 11B: DNA and amino acid sequences (SEQ. ID NOS: 16 and 17) of the first version ("a") of reshaped human 21.6 heavy chain variable region.

FIG. 12: ELISA comparison of chimeric and reshaped human 21.6 antibodies to bind to L cells expressing human $\alpha4\beta1$ integrin on their surface.

FIG. 13: Comparison of mouse 21.6 antibody with a different anti-VLA-4 antibody, L25. Panel A compares the ability of the antibodies to block binding of U937 monocytic cells to purified VCA-1 in the presence and absence of Mn^{2+}. Panel B compares the ability of the antibodies to block binding of Jurkat cells to increasing concentrations of VCAM-1.

FIG. 14: Delay of weight loss in animals treated with mouse or human 21.6 antibody.

FIG. 15: Reversal of clinical symptoms in animals treated with mouse or human 21.6 antibody.

FIG. 16: Reversal of weight loss in animals treated with mouse or human 21.6 antibody.

DEFINITIONS

Abbreviations for the twenty naturally occurring amino acids follow conventional usage (Immunology—A Synthesis (2nd ed., E. S. Golub & D. R. Gren, eds., Sinauer Associates, Sunderland, Mass., 1991)). Stereoisomers (e.g., D-amino acids) of the twenty conventional amino acids, unnatural amino acids such as α,α-disubstituted amino acids, N-alkyl amino acids, lactic acid, and other unconven-

tional amino acids may also be suitable components for polypeptides of the present invention. Examples of unconventional amino acids include: 4-hydroxyproline, γ-carboxyglutamate, ε-N,N,N-trimethyllysine, ε-N-acetyllysine, O-phosphoserine, N-acetylserine, N-formylmethionine, 3-methylhistidine, 5-hydroxylysine, ω-N-methylarginine, and other similar amino acids and imino acids (e.g., 4-hydroxyproline). Moreover, amino acids may be modified by glycosylation, phosphorylation and the like.

In the polypeptide notation used herein, the lefthand direction is the amino terminal direction and the righthand direction is the carboxy-terminal direction, in accordance with standard usage and convention. Similarly, unless specified otherwise, the lefthand end of single-stranded polynucleotide sequences is the 5' end; the lefthand direction of double-stranded polynucleotide sequences is referred to as the 5' direction. The direction of 5' to 3' addition of nascent RNA transcripts is referred to as the transcription direction; sequence regions on the DNA strand having the same sequence as the RNA and which are 5' to the 5' end of the RNA transcript are referred to as "upstream sequences"; sequence regions on the DNA strand having the same sequence as the RNA and which are 3' to the 3' end of the RNA transcript are referred to as "downstream sequences."

The phrase "polynucleotide sequence" refers to a single or double-stranded polymer of deoxyribonucleotide or ribonucleotide bases read from the 5' to the 3' end. It includes self-replicating plasmids, infectious polymers of DNA or RNA and non-functional DNA or RNA.

The following terms are used to describe the sequence relationships between two or more polynucleotides: "reference sequence", "comparison window", "sequence identity", "percentage of sequence identity", and "substantial identity". A "reference sequence" is a defined sequence used as a basis for a sequence comparison; a reference sequence may be a subset of a larger sequence, for example, as a segment of a full-length CDNA or gene sequence given in a sequence listing, such as a polynucleotide sequence of FIGS. 1 or 2, or may comprise a complete DNA or gene sequence. Generally, a reference sequence is at least 20 nucleotides in length, frequently at least 25 nucleotides in length, and often at least 50 nucleotides in length. Since two polynucleotides may each (1) comprise a sequence (i.e., a portion of the complete polynucleotide sequence) that is similar between the two polynucleotides, and (2) may further comprise a sequence that is divergent between the two polynucleotides, sequence comparisons between two (or more) polynucleotides are typically performed by comparing sequences of the two polynucleotides over a "comparison window" to identify and compare local regions of sequence similarity. A "comparison window", as used herein, refers to a conceptual segment of at least 20 contiguous nucleotide positions wherein a polynucleotide sequence may be compared to a reference sequence of at least 20 contiguous nucleotides and wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) of 20 percent or less as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. Optimal alignment of sequences for aligning a comparison window may be conducted by the local homology algorithm of Smith & Waterman, *Adv. Appl. Math.* 2:482 (1981), by the homology alignment algorithm of Needleman & Wunsch, *J. Mol. Biol.* 48:443 (1970), by the search for similarity method of Pearson & Lipman, *Proc. Natl. Acad. Sci.* (USA) 85:2444 (1988) (each of which is incorporated by reference in its entirety for all purposes), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package Release 7.0, Genetics Computer Group, 575 Science Dr., Madison, Wis.), or by inspection, and the best alignment (i.e., resulting in the highest percentage of sequence similarity over the comparison window) generated by the various methods is selected. The term "sequence identity" means that two polynucleotide sequences are identical (i.e., on a nucleotide-by-nucleotide basis) over the window of comparison. The term "percentage of sequence identity" is calculated by comparing two optimally aligned sequences over the window of comparison, determining the number of positions at which the identical nucleic acid base (e.g., A, T, C, G, U, or I) occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison (i.e., the window size), and multiplying the result by 100 to yield the percentage of sequence identity. The terms "substantial identity" as used herein denotes a characteristic of a polynucleotide sequence, wherein the polynucleotide comprises a sequence that has at least 85 percent sequence identity, preferably at least 90 to 95 percent sequence identity, more usually at least 99 percent sequence identity as compared to a reference sequence over a comparison window of at least 20 nucleotide positions, frequently over a window of at least 25–50 nucleotides, wherein the percentage of sequence identity is calculated by comparing the reference sequence to the polynucleotide sequence which may include deletions or additions which total 20 percent or less of the reference sequence over the window of comparison. The reference sequence may be a subset of a larger sequence, for example, the sequence shown in FIGS. 1 or 2.

As applied to polypeptides, the term "sequence identity" means peptides share identical amino acids at corresponding positions. The term "sequence similarity" means peptides have identical or similar amino acids (i.e., conservative substitutions) at corresponding positions. The term "substantial identity" means that two peptide sequences, when optimally aligned, such as by the programs GAP or BESTFIT using default gap weights, share at least 80 percent sequence identity, preferably at least 90 percent sequence identity, more preferably at least 95 percent sequence identity or more (e.g., 99 percent sequence identity). Preferably, residue positions which are not identical differ by conservative amino acid substitutions. The term "substantial similarity" means that two peptide sequences share corresponding percentages of sequence similarity.

The term "substantially pure" means an object species is the predominant species present (i.e., on a molar basis it is more abundant than any other individual species in the composition), and preferably a substantially purified fraction is a composition wherein the object species comprises at least about 50 percent (on a molar basis) of all macromolecular species present. Generally, a substantially pure composition will comprise more than about 80 to 90 percent of all macromolecular species present in the composition. Most preferably, the object species is purified to essential homogeneity (contaminant species cannot be detected in the composition by conventional detection methods) wherein the composition consists essentially of a single macromolecular species.

For purposes of classifying amino acids substitutions as conservative or nonconservative, amino acids are grouped as follows: Group I (hydrophobic sidechains): norleucine, met, ala, val, leu, ile; Group II (neutral hydrophilic side chains):

cys, ser, thr; Group III (acidic side chains): asp, glu; Group IV (basic side chains): asn, gln, his, lys, arg; Group V (residues influencing chain orientation): gly, pro; and Group VI (aromatic side chains): trp, tyr, phe. Conservative substitutions involve substitutions between amino acids in the same class. Non-conservative substitutions constitute exchanging a member of one of these classes for another.

Amino acids from the variable regions of the mature heavy and light chains of immunoglobulins are designated Hx and Lxx respectively, where x is a number designating the position of an amino acids according to the scheme of Kabat et al., *Sequences of Proteins of Immunological Interest* (National Institutes of Health, Bethesda, Md. (1987) and (1991)) (hereinafter collectively referred to as "Kabat et al.," incorporated by reference in their entirety for all purposes). Kabat et al. list many amino acid sequences for antibodies for each subclass, and list the most commonly occurring amino acid for each residue position in that subclass. Kabat et al. use a method for assigning a residue number to each amino acid in a listed sequence, and this method for assigning residue numbers has become standard in the field. Kabat et al.'s scheme is extendible to other antibodies not included in the compendium by aligning the antibody in question with one of the consensus sequences in Kabat et al. The use of the Kabat et al. numbering system readily identifies amino acids at equivalent positions in different antibodies. For example, an amino acid at the L50 position of a human antibody occupies the equivalence position to an amino acid position L50 of a mouse antibody.

DETAILED DESCRIPTION

I. Humanized Antibodies Specific for VLA-4

In one embodiment of the invention, humanized immunoglobulins (or antibodies) specific for the alpha-4 subunit of VLA-4 are provided. The humanized immunoglobulins have variable framework regions substantially from a human immunoglobulin (termed an acceptor immunoglobulin) and complementarity determining regions substantially from a mouse immunoglobulin termed mu MAb 21.6 (referred to as the donor immunoglobulin). The constant region(s), if present, are also substantially from a human immunoglobulin. The humanized antibodies exhibit a specific binding affinity for VLA-4 of at least 10^7, 10^8, 10^9, or $10^{10}M^{-1}$. Usually the upper limit of binding affinity of the humanized antibodies for VLA-4 is within a factor of three or five of that of mu MAb 21.6 (about 10^9M^{-1}). Often the lower limit of binding affinity is also within a factor of three or five of that of mu MAb 21.6.

A. General Characteristics of Immunoglobulins

The basic antibody structural unit is known to comprise a tetramer. Each tetramer is composed of two identical pairs of polypeptide chains, each pair having one "light" (about 25 kDa) and one "heavy" chain (about 50–70 kDa). The amino-terminal portion of each chain includes a variable region of about 100 to 110 or more amino acids primarily responsible for antigen recognition. The carboxy-terminal portion of each chain defines a constant region primarily responsible for effector function.

Light chains are classified as either kappa or lambda. Heavy chains are classified as gamma, mu, alpha, delta, or epsilon, and define the antibody's isotype as IgG, IgM, IgA, IgD and IgE, respectively. Within light and heavy chains, the variable and constant regions are joined by a "J" region of about 12 or more amino acids, with the heavy chain also including a "D" region of about 10 more amino acids. (See generally, *Fundamental Immunology* (Paul, W., ed., 2nd ed. Raven Press, N.Y., 1989), Ch. 7 (incorporated by reference in its entirety for all purposes).

The variable regions of each light/heavy chain pair form the antibody binding site. The chains all exhibit the same general structure of relatively conserved framework regions (FR) joined by three hypervariable regions, also called complementarity determining regions or CDRs. The CDRs from the two chains of each pair are aligned by the framework regions, enabling binding to a specific epitope. CDR and FR residues are delineated according to the standard sequence definition of Kabat et al., supra. An alternative structural definition has been proposed by Chothia et al., *J. Mol. Biol.* 196:901–917 (1987); *Nature* 342:878–883 (1989); and *J. Mol. Biol.* 186:651–663 (1989) (hereinafter collectively referred to as "Chothia et al." and incorporated by reference in their entirety for all purposes). When framework positions, as defined by Kabat et al., supra, that constitute structural loop positions as defined by Chothia et al., supra, the amino acids present in the mouse antibody are usually incorporated into the humanized antibody.

B. Production of Humanized Antibodies

(1) Mouse MAb 21.6

The starting material for production of humanized antibodies is mu MAb 21.6. The isolation and properties of this antibody are described in U.S. Ser. No. 07/871,223. Briefly, mu MAb 21.6 is specific for the alpha-4 subunit of VLA-4 and has been shown to inhibit human lymphocyte binding to tissue cultures of rat brain cells stimulated with tumor necrosis factor. The cloning and sequencing of CDNA encoding the mu MAb 21.6 antibody heavy and light chain variable regions is described in Example 1, and the nucleotide and predicted amino acids sequences are shown in FIGS. **1** and **2**. These figures also illustrate the subdivision of the amino acid coding sequencing into framework and complementarity determining domains. From N-terminal to C-terminal, both light and heavy chains comprise the domains FR1, CDR1, FR2, CDR2, FR3, CDR3 and FR4. The assignment of amino acids to each domain is in accordance with the numbering convention of Kabat et al., supra.

(2) Selection of Human Antibodies to Supply Framework Residues

The substitution of mouse CDRs into a human variable domain framework is most likely to result in retention of their correct spatial orientation if the human variable domain framework adopts the same or similar conformation to the mouse variable framework from which the CDRs originated. This is achieved by obtaining the human variable domains from human antibodies whose framework sequences exhibit a high degree of sequence identity with the murine variable framework domains from which the CDRs were derived. The heavy and light chain variable framework regions can be derived from the same or different human antibody sequences. The human antibody sequences can be the sequences of naturally occurring human antibodies or can be consensus sequences of several human antibodies. See Kettleborough et al., *Protein Engineering* 4:773 (1991); Kolbinger et al., *Protein Engineering* 6:971 (1993).

Suitable human antibody sequences are identified by computer comparisons of the amino acid sequences of the mouse variable regions with the sequences of known human antibodies. The comparison is performed separately for heavy and light chains but the principles are similar for each. This comparison reveals that the mu 21.6 light chain shows greatest sequence identity to human light chains of subtype kappa 1, and that the mu 21.6 heavy chain shows greatest sequence identity to human heavy chains of subtype one, as defined by Kabat et al., supra. Thus, light and heavy human framework regions are usually derived from human antibodies of these subtypes, or from consensus sequences of

such subtypes. The preferred light and heavy chain human variable regions showing greatest sequence identity to the corresponding regions from mu MAb 21.6 are from antibodies RE1 and 21/28'CL respectively.

(3) Computer Modelling

The unnatural juxtaposition of murine CDR regions with human variable framework region can result in unnatural conformational restraints, which, unless corrected by substitution of certain amino acid residues, lead to loss of binding affinity. The selection of amino acid residues for substitution is determined, in part, by computer modelling. Computer hardware and software for producing three-dimensional images of immunoglobulin molecules are widely available. In general, molecular models are produced starting from solved structures for immunoglobulin chains or domains thereof. The chains to be modelled are compared for amino acid sequence similarity with chains or domains of solved three dimensional structures, and the chains or domains showing the greatest sequence similarity is/are selected as starting points for construction of the molecular model. For example, for the light chain of mu MAb 21.6, the starting point for modelling the framework regions, CDR1 and CDR2 regions, was the human light chain RE1. For the CDR3 region, the starting point was the CDR3 region from the light chain of a different human antibody HyHEL-5. The solved starting structures are modified to allow for differences between the actual amino acids in the immunoglobulin chains or domains being modelled, and those in the starting structure. The modified structures are then assembled into a composite immunoglobulin. Finally, the model is refined by energy minimization and by verifying that all atoms are within appropriate distances from one another and that bond lengths and angles are within chemically acceptable limits. Example 4 discusses in more detail the steps taken to produce a three dimensional computer model for the variable regions of the mu MAb 21.6, and the model is shown in FIG. 5. This model can in turn serve as a starting point for predicting the three-dimensional structure of an antibody containing the mu MAb 21.6 complementarity determining regions substituted in human framework structures. Additional models can be constructed representing the structure when further amino acid substitutions to be discussed infra, are introduced.

(4) Substitution of Amino Acid Residues

As noted supra, the humanized antibodies of the invention comprise variable framework regions substantially from a human immunoglobulin and complementarity determining regions substantially from a mouse immunoglobulin termed mu MAb 21.6. Having identified the complementarity determining regions of mu MAb 21.6 and appropriate human acceptor immunoglobulins, the next step is to determine which, if any, residues from these components should be substituted to optimize the properties of the resulting humanized antibody. In general, substitution of human amino acid residues with murine should be minimized, because introduction of murine residues increases the risk of the antibody eliciting a HAMA response in humans. Amino acids are selected for substitution based on their possible influence on CDR conformation and/or binding to antigen. Investigation of such possible influences is by modelling, examination of the characteristics of the amino acids at particular locations, or empirical observation of the effects of substitution or mutagenesis of particular amino acids.

When an amino acid differs between a mu MAb 21.6 variable framework region and an equivalent human variable framework region, the human framework amino acid should usually be substituted by the equivalent mouse amino acid if it is reasonably expected that the amino acid:

(1) noncovalently binds antigen directly (e.g., amino acids at positions L49, L69 of mu MAb 21.6),

(2) is adjacent to a CDR region, is part of a CDR region under the alternative definition proposed by Chothia et al., supra, or otherwise interacts with a CDR region (e.g., is within about 3 Å of a CDR region) (e.g., amino acids at positions L45, L58, H27, H28, H29, H30 and H71 of mu MAb 21.6), or

(3) participates in the V_L-V_H interface (e.g., amino acids at position H44 of mu MAb 21.6).

Other candidates for substitution are acceptor human framework amino acids that are unusual for a human immunoglobulin at that position (e.g., amino acids at positions L104, L105 and L107 of mu MAb 21.6). These amino acids can be substituted with amino acids from the equivalent position of more typical human immunoglobulins. Alternatively, amino acids from equivalent positions in the mouse MAb 21.6 can be introduced into the human framework regions when such amino acids are typical of human immunoglobulin at the equivalent positions.

In general, substitution of all or most of the amino acids fulfilling the above criteria is desirable. Occasionally, however, there is some ambiguity about whether a particular amino acid meets the above criteria, and alternative variant immunoglobulins are produced, one of which has that particular substitution, the other of which does not. The humanized antibodies of the present invention will usually contain a substitution of a human light chain framework residue with a corresponding mu MAb 21.6 residue in at least 1, 2 or 3, and more usually 4, of the following positions: L45, L49, L58 and L69. The humanized antibodies also usually contain a substitution of a human heavy chain framework residue in at least 1, 2, 3, 4, or 5, and sometimes 6, of the following positions: H27, H28, H29, H30, H44 and H71. Optionally, H36 may also be substituted. In preferred embodiments when the human light chain acceptor immunoglobulin is RE1, the light chain also contains substitutions in at least 1 or 2, and more usually 3, of the following positions: L104, L105 and L107 These positions are substituted with the amino acid from the equivalent position of a human immunoglobulin having a more typical amino acid residues. Appropriate amino acids to substitute are shown in FIGS. 6 and 7.

Usually the CDR regions in humanized antibodies are substantially identical, and more usually, identical to the corresponding CDR regions in the mu MAb 21.6 antibody. Occasionally, however, it is desirable to change one of the residues in a CDR region. For example, Example 5 identifies an amino acid similarity between the mu MAb 21.6 CDR3 and the VCAM-1 ligand. This observation suggests that the binding affinity of humanized antibodies might be improved by redesigning the heavy chain CDR3 region to resemble VCAM-1 even more closely. Accordingly, one or more amino acids from the CDR3 domain can be substituted with amino acids from the VCAM-1 binding domain. Although not usually desirable, it is sometimes possible to make one or more conservative amino acid substitutions of CDR residues without appreciably affecting the binding affinity of the resulting humanized immunoglobulin.

Other than for the specific amino acid substitutions discussed above, the framework regions of humanized immunoglobulins are usually substantially identical, and more usually, identical to the framework regions of the human antibodies from which they were derived. Of course, many of the amino acids in the framework region make little or no direct contribution to the specificity or affinity of an antibody. Thus, many individual conservative substitutions of

framework residues can be tolerated without appreciable change of the specificity or affinity of the resulting humanized immunoglobulin. However, in general, such substitutions are undesirable.

(5) Production of Variable Regions

Having conceptually selected the CDR and framework components of humanized immunoglobulins, a variety of methods are available for producing such immunoglobulins. Because of the degeneracy of the code, a variety of nucleic acid sequences will encode each immunoglobulin amino acid sequence. The desired nucleic acid sequences can be produced by de novo solid-phase DNA synthesis or by PCR mutagenesis of an earlier prepared variant of the desired polynucleotide. Oligonucleotide-mediated mutagenesis is a preferred method for preparing substitution, deletion and insertion variants of target polypeptide DNA. See Adelman et al., DNA 2:183 (1983). Briefly, the target polypeptide DNA is altered by hybridizing an oligonucleotide encoding the desired mutation to a single-stranded DNA template. After hybridization, a DNA polymerase is used to synthesize an entire second complementary strand of the template that incorporates the oligonucleotide primer, and encodes the selected alteration in the target polypeptide DNA.

(6) Selection of Constant Region

The variable segments of humanized antibodies produced as described supra are typically linked to at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. Human constant region DNA sequences can be isolated in accordance with well-known procedures from a variety of human cells, but preferably immortalized B-cells (see Kabat et al., supra, and W087/02671) (each of which is incorporated by reference in its entirety for all purposes). Ordinarily, the antibody will contain both light chain and heavy chain constant regions. The heavy chain constant region usually includes CH1, hinge, CH2, CH3, and CH4 regions.

The humanized antibodies include antibodies having all types of constant regions, including IgM, IgG, IgD, IgA and IgE, and any isotype, including IgG1, IgG2, IgG3 and IgG4. When it is desired that the humanized antibody exhibit cytotoxic activity, the constant domain is usually a complement-fixing constant domain and the class is typically IgG_1. When such cytotoxic activity is not desirable, the constant domain may be of the IgG_2 class. The humanized antibody may comprise sequences from more than one class or isotype.

(7) Expression Systems

Nucleic acids encoding humanized light and heavy chain variable regions, optionally linked to constant regions, are inserted into expression vectors. The light and heavy chains can be cloned in the same or different expression vectors. The DNA segments encoding immunoglobulin chains are operably linked to control sequences in the expression vector(s) that ensure the expression of immunoglobulin polypeptides. Such control sequences include a signal sequence, a promoter, an enhancer, and a transcription termination sequence. Expression vectors are typically replicable in the host organisms either as episomes or as an integral part of the host chromosomal DNA. Commonly, expression vectors will contain selection markers, e.g., tetracycline or neomycin, to permit detection of those cells transformed with the desired DNA sequences (see, e.g., U.S. Pat. No. 4,704,362.)

E. coli is one prokaryotic host useful particularly for cloning the polynucleotides of the present invention. Other microbial hosts suitable for use include bacilli, such as Bacillus subtilus, and other enterobacteriaceae, such as Salmonella, Serratia, and various Pseudomonas species. In these prokaryotic hosts, one can also make expression vectors, which will typically contain expression control sequences compatible with the host cell (e.g., an origin of replication). In addition, any number of a variety of well-known promoters will be present, such as the lactose promoter system, a tryptophan (trp) promoter system, a beta-lactamase promoter system, or a promoter system from phage lambda The promoters will typically control expression, optionally with an operator sequence, and have ribosome binding site sequences and the like, for initiating and completing transcription and translation.

Other microbes, such as yeast, may also be used for expression. Saccharomyces is a preferred host, with suitable vectors having expression control sequences, such as promoters, including 3-phosphoglycerate kinase or other glycolytic enzymes, and an origin of replication, termination sequences and the like as desired.

In addition to microorganisms, mammalian tissue cell culture may also be used to express and produce the polypeptides of the present invention (see Winnacker, *From Genes to Clones* (VCH Publishers, N.Y., N.Y., 1987). Eukaryotic cells are actually preferred, because a number of suitable host cell lines capable of secreting intact immunoglobulins have been developed in the art, and include the CHO cell lines, various Cos cell lines, HeLa cells, preferably myeloma cell lines, or transformed B-cells or hybridomas. Expression vectors for these cells can include expression control sequences, such as an origin of replication, a promoter, and an enhancer (Queen et al., *Immunol.* Rev. 89:49–68 (1986)), and necessary processing information sites, such as ribosome binding sites, RNA splice sites, polyadenylation sites, and transcriptional terminator sequences. Preferred expression control sequences are promoters derived from immunoglobulin genes, SV40, adenovirus, bovine papilloma virus, cytomegalovirus and the like.

The vectors containing the polynucleotide sequences of interest (e.g., the heavy and light chain encoding sequences and expression control sequences) can be transferred into the host cell by well-known methods, which vary depending on the type of cellular host. For example, calcium chloride transfection is commonly utilized for prokaryotic cells, whereas calcium phosphate treatment or electroporation may be used for other cellular hosts. (See generally Sambrook et al., *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Press, 2nd ed., 1989) (incorporated by reference in its entirety for all purposes). When heavy and light chains are cloned on separate expression vectors, the vectors are co-transfected to obtain expression and assembly of intact immunoglobulins.

Once expressed, the whole antibodies, their dimers, individual light and heavy chains, or other immunoglobulin forms of the present invention can be purified according to standard procedures of the art, including ammonium sulfate precipitation, affinity columns, column chromatography, gel electrophoresis and the like (see generally *Scopes, Protein Purification* (Springer-Verlag, N.Y., 1982). Substantially pure immunoglobulins of at least about 90 to 95% homogeneity are preferred, and 98 to 99% or more homogeneity most preferred, for pharmaceutical uses.

C. Fragments of Humanized Antibodies

In another embodiment of the invention, fragments of humanized antibodies are provided. Typically, these fragments exhibit specific binding to the VLA-4 antigen with an affinity of at least 10^7M^{-1}, and more typically 10^8 or 10^9M^{-1}. Humanized antibody fragments include separate

heavy chains, light chains Fab, Fab' $F(ab')_2$, Fabc, and Fv. Fragments are produced by recombinant DNA techniques, or by enzymic or chemical separation of intact immunoglobulins.

II. Nucleic Acids

The humanized antibodies and fragments thereof are usually produced by expression of nucleic acids. All nucleic acids encoding a humanized antibody or a fragment thereof described in this application are expressly included in the invention.

III. Computers

In another aspect of the invention, computers programmed to display three dimensional images of antibodies on a monitor are provided. For example, a Silicon Graphics IRIS 4D workstation running under the UNIX operating system and using the molecular modelling package QUANTA (Polygen Corp. USA) is suitable. Computers are useful for visualizing models of variants of humanized antibodies. In general, the antibodies of the invention already provide satisfactory binding affinity. However, it is likely that antibodies with even stronger binding affinity could be identified by further variation of certain amino acid residues. The three dimensional image will also identify many noncritical amino acids, which could be the subject of conservative substitutions without appreciable affecting the binding affinity of the antibody. Collectively even conservative substitutions can have a significant effect on the properties of an immunoglobulin. However, it is likely many individual conservative substitutions will not significantly impair the properties of the immunoglobulins.

IV. Testing Humanized Antibodies

The humanized antibodies of the invention are tested by a variety of assays. These include a simple binding assay for detecting the existence or strength of binding of an antibody to cells bearing the VLA-receptor. The antibodies are also tested for their capacity to block the interaction of cells bearing the VLA-4 receptor with endothelial cells expressing a VCAM-1 ligand. The endothelial cells may be grown and stimulated in culture or may be a component of naturally occurring brain tissue sections. See Yednock et al., supra, and U.S. Ser. No. 07/871,223. The humanized antibodies are also tested for their capacity to prevent or reduce inflammation and subsequent paralysis in laboratory animals having experimental autoimmune encephalomyelitis (EAE). EAE is induced by injection of a laboratory animal with $CD4^+$ T-cells specific for myelin basic protein or by directly immunizing animals with myelin basic protein. This protein is localized in the central nervous system, and the reactive T-cells initiate destruction of sheaths containing this protein in a manner that simulates the autoimmune response in multiple sclerosis. See Yednock et al., supra, and copending U.S. Ser. No. 07/871,223.

V. Pharmaceutical Compositions

The invention provides pharmaceutical compositions to be used for prophylactic or therapeutic treatment comprising an active therapeutic agent, i.e., a humanized 21.6 antibody or a binding fragment thereof, and a variety of other components. The preferred form depends on the intended mode of administration and therapeutic application. The compositions can also include, depending on the formulation desired, pharmaceutically-acceptable, non-toxic carriers or diluents, which are defined as vehicles commonly used to formulate pharmaceutical compositions for animal or human administration. The diluent is selected so as not to affect the biological activity of the combination. Examples of such diluents are distilled water, physiological phosphate-buffered saline, Ringer's solutions, dextrose solution, and Hank's solution. In addition, the pharmaceutical composition or formulation may also include other carriers, adjuvants, or nontoxic, nontherapeutic, nonimmunogenic stabilizers and the like.

For parenteral administration, the antibodies of the invention can be administered as injectionable dosages of a solution or suspension of the substance in a physiologically acceptable diluent with a pharmaceutical carrier which can be a sterile liquid such as water and oils with or without the addition of a surfactant and other pharmaceutically preparations are those of petroleum, animal, vegetable, or synthetic origin, for example, peanut oil, soybean oil, and mineral oil. In general, glycols such as propylene glycol or polyethylene glycol are preferred liquid carriers, particularly for injectable solutions. The antibodies of this invention can be administered in the form of a depot injection or implant preparation which can be formulated in such a manner as to permit a sustained release of the active ingredient. A preferred composition comprises monoclonal antibody at 5 mg/mL, formulated in aqueous buffer consisting of 50 mM L-histidine, 150 mM NaCl, adjusted to pH 6.0 with HCl.

VI. Methods of Diagnosis

The humanized antibodies and their binding fragments are useful for detecting the presence of cells bearing the VLA-4 receptor. The presence of such cells in the brain is diagnostic of an inflammatory response and may signal the need for commencement of a therapeutic method discussed infra. Diagnosis can be accomplished by removing a cellular sample from a patient. The amount of expressed VLA-4 antigen in individual cells of the sample is then determined, e.g., by immunohistochemical staining of fixed cells or by Western blotting of a cell extract with a humanized MAb 21.6 antibody or a binding fragment thereof.

Diagnosis can also be achieved by in vivo administration of a labelled humanized MAb 21.6 (or binding fragment) and detection by in vivo imaging. The concentration of humanized MAb 21.6 administered should be sufficient that the binding to cells having the target antigen is detectable compared to the background signal. The diagnostic reagent can be labelled with a radioisotope for camera imaging, or a paramagnetic isotope for magnetic resonance or electron spin resonance imaging.

A change (typically an increase) in the level of VLA-4 protein in a cellular sample or imaged from an individual, which is outside the range of clinically established normal levels, may indicate the presence of an undesirable inflammatory response reaction in the individual from whom the sample was obtained, and/or indicate a predisposition of the individual for developing (or progressing through) such a reaction. VLA-4 protein can also be employed as a differentiation marker to identify and type cells of certain lineages and developmental origins. Such cell-type specific detection can be used for histopathological diagnosis of undesired immune responses.

VII. Methods of Treatment

The invention also provides methods of treatment that exploit the capacity of humanized MAb 21.6 to block α4-dependent interactions of the VLA-4 receptor. The α4-dependent interaction of the VLA-4 receptor with the VCAM-1 ligand on endothelial cells is an early event in many inflammatory responses, particularly those of the central nervous system. Undesired diseases and conditions resulting from inflammation of the central nervous system having acute clinical exacerbations include multiple sclerosis (Yednock et al., *Nature* 356, 63 (1992); Baron et al., *J. Exp. Med.* 177, 57 (1993)), meningitis, encephalitis, stroke, other cerebral traumas, inflammatory bowel disease

(Hamann et al., *J. Immunol.* 152, 3238 (1994)), ulcerative colitis, Crohn's disease, rheumatoid arthritis (van Dinther-Janssen et al., *J. Immunol.* 147, 4207 (1991); van Dinther-Janssen et al., *Annals Rheumatic* Diseases 52, 672 (1993); Elices et al., *J. Clin. Invest.* 93, 405 (1994); Postigo et al., *J. Clin. Invest.* 89, 1445 (1992), asthma (Mulligan et al., *J. Immunol.* 150, 2407 (1993)) and acute juvenile onset diabetes (Type 1) (Yang et al., PNAS 90, 10494 (1993); Burkly et al., Diabetes 43, 529 (1994); Baron et al., *J. Clin. Invest.* 93, 1700 (1994)).

Multiple sclerosis is a progressive neurological autoimmune disease that affects an estimated 250,000 to 350,000 people in the United States. Multiple sclerosis is thought to be a the result of a specific autoimmune reaction in which certain leukocytes attack and initiate the destruction of myelin, the insulating sheath covering nerve fibers. In an animal model for multiple sclerosis, murine monoclonal antibodies directed against alpha-4-beta-1 integrin have been shown to block the adhesion of leukocytes to the endothelium, and thus prevent inflammation of the central nervous system and subsequent paralysis in the animals.

The humanized MAb 21.6 antibodies of the present invention offer several advantages over the mouse antibodies already shown to be effective in animals models:

1) The human immune system should not recognize the framework or constant region of the humanized antibody as foreign, and therefore the antibody response against such an injected antibody should be less than against a totally foreign mouse antibody or a partially foreign chimeric antibody.
2) Because the effector portion of the humanized antibody is human, it may interact better with other parts of the human immune system.
3) Injected mouse antibodies have been reported to have a half-life in the human circulation much shorter than the half-life of normal human antibodies (Shaw et al., *J. Immunol.* 138:4534–4538 (1987)). Injected humanized antibodies have a half-life essentially equivalent to naturally occurring human antibodies, allowing smaller and less frequent doses.

The pharmaceutical compositions discussed supra can be administered for prophylactic and/or therapeutic treatments of multiple sclerosis or other inflammatory disorders, particularly those of the central nervous system. In therapeutic applications, compositions are administered to a patient suspected of, or already suffering from a disease such as multiple sclerosis, in an amount sufficient to cure, or at least partially arrest, the symptoms of the disease and its complications. An amount adequate to accomplish this is defined as a therapeutically- or pharmaceutically-effective dose.

In prophylactic applications, pharmaceutical compositions are administered to a patient susceptible to, or otherwise at risk of, a particular disease in an amount sufficient to eliminate or reduce the risk or delay the outset of the disease. Such an amount is defined to be a prophylactically effective dose. In patients with multiple sclerosis in remission, risk may be assessed by NMR imaging or, in some cases, by presymptomatic indications observed by the patient.

The pharmaceutical compositions will be administered by parenteral, topical, intravenous, oral, or subcutaneous, intramuscular local administration, such as by aerosol or transdermally, for prophylactic and/or therapeutic treatment. Although the proteinaceous substances of this invention may survive passage through the gut following oral administration, subcutaneous, intravenous, intramuscular, intraperitoneal administration by depot injection; or by implant preparation. are preferred.

The pharmaceutical compositions can be administered in a variety of unit dosage forms depending upon the method of administration. For example, unit dosage forms suitable for oral administration include powder, tablets, pills, capsules, and lozenges.

Effective doses of the compositions of the present invention, for the treatment of the above described conditions will vary depending upon many different factors, including means of administration, target site, physiological state of the patient, and other medicants administered. Thus, treatment dosages will need to be titrated to optimize safety and efficacy. These compositions may be administered to mammals for veterinary use and for clinical use in humans in a manner similar to other therapeutic agents, i.e., in a physiologically acceptable carrier. In general, the administration dosage will range from about 0.0001 to 100 mg/kg, and more usually 0.01 to 0.5 mg/kg of the host body weight.

In a preferred treatment regime, the antibody is administered by intravenous infusion or subcutaneous injection at a dose from 1 to 5 mg antibody per kilo of bodyweight. The dose is repeated at interval from 2 to 8 weeks. Within this range, the preferred treatment regimen is 3 mg antibody per kilo of bodyweight repeated at a 4 week interval.

VIII. Other Uses

The humanized antibodies are also useful for affinity purification of the VLA-4 receptor. The antibodies are immobilized to a solid support and a solution of dispersed proteins is passed over the support. VLA-4 binds to the support and is thereby separated from other proteins. The purified VLA-4 or a fragment thereof, made available by this method, can be used as a vaccine or as an immunogen for producing further antibodies.

The humanized antibodies of the invention are also useful for generating idiotypic antibodies by, for example, immunization of an animal with a humanized antibody. An anti-idiotype antibody whose binding to the human antibody is inhibited by VLA-4 or fragments thereof is selected. Because both the anti-idiotypic antibody and the VLA-4 or fragments thereof bind to the humanized immunoglobulin, the anti-idiotypic antibody may represent the "internal image" of an epitope and thus may substitute the ligand of the VLA-4 receptor, i.e., VCAM-1.

EXAMPLES

Example 1

Cloning and Sequencing of the Mouse 21.6 Variable Regions

The mouse anti-VLA antibody 21.6 has been described in co-pending application U.S. Ser. No. 07/871,223. Total RNA was isolated from hybridoma cells producing mouse 21.6 antibody. First-strand cDNA was synthesized using a kit (Pharmacia Biosystems Limited). Heavy and light chain variable regions were obtained by using PCR primers designed to hybridize to sequences flanking and external to the sequences coding for the variable regions, thereby allowing cloning of the entire coding sequences for the mouse 21.6 antibody variable regions. Sense PCR primers hybridizing to the 5'-ends of mouse kappa light-chain leader sequences and of mouse heavy-chain leader sequences were designed based on databases of 42 mouse kappa light-chain leader sequences and of 55 mouse heavy-chain leader sequences (Jones & Bendig, Bio/Technology 9:88–89 (1991) (incorporated by reference in its entirety for all purposes)). These primers were used in conjunction with anti-sense PCR primers hybridizing to the 3'-ends of the mouse constant regions (kappa or gamma).

Mouse 21.6 kappa V_L regions were PCR-amplified in a 50 μl reaction typically containing 10 mM Tris-HCl (pH 8.3),

50 mM KCl, 200 μM dNTPs, 1.5 mM $MgCl_2$, 1 unit of AmpliTaq (Perkin Elmer Cetus) DNA polymerase, 1 μl of CDNA template, 0.25 μM of MKV primer and 0.25 μM of mouse kappa light chain anti-sense PCR primer (FIG. 1). Mouse 21.6 V_H regions were PCR-amplified as described above except that MHVH primer and an anti-sense PCR primer specific for the mouse IgG1 heavy chain constant region were used (FIG. 2). Each PCR reaction was cycled, after an initial melt at 94° C. for 5 min, at 94° C. for 1 min, 55° C. for 1 min, and 72° C. for 2 min over 25 cycles. The completion of the last cycle was followed by a final extension at 72° C. for 10 min. The ramp time between the primer-annealing and extension steps was 2.5 min. Following PCR amplification, 10 μl aliquots from each reaction were analyzed on ethidium-bromide-stained 1.5% agarose gels.

The PCR products were cloned using the "TA Cloning System" (Invitrogen Corporation). Vectors containing inserts of the correct size were sequenced using double-stranded plasmid DNA and Sequenase (United States Biochemical Corporation). To avoid any errors that might have been introduced during the PCR amplification steps, at least two independently PCR-amplified and cloned DNA fragments were sequenced for each variable region.

The sequences of PCR products were compared with other mouse light chain and heavy chain variable regions (see Tables 1 and 2). This comparison indicated that the PCR products from MKV2 and MKV4 primers represent authentic mouse 21.6 kappa variable regions, and those from MHV1 and MHV2 primers represent authentic mouse V_H regions, and it was concluded that the sequences of these product are those of the mouse 21.6 antibody variable regions. The DNA and amino acid sequences of the cDNA coding for the mouse 21.6 V_L and V_H regions are shown in FIGS. 1 and 2.

TABLE 1

Comparison of the mouse 21.6 light chain variable region to other light chain variable regions.

Mouse 21.6 V_L versus:	Percent Similarity[1]	Percent Identity
Consensus sequence for mouse kappa V_L subgroup 5[2]	84.0	72.6
Consensus sequence for human kappa V_L subgroup 1[2]	84.0	69.8
Consensus sequence for human kappa V_L subgroup 2[2]	65.1	52.8
Consensus sequence for human kappa V_L subgroup 3[2]	72.6	57.5
Consensus sequence for human kappa V_L subgroup 4[2]	72.6	58.5
Sequence of V_L from human REI[3] (Member of human kappa V_L subgroup 1)	81.0	72.4

[1]Percent similarity was determined using the "GAP" program of the University of Wisconsin Genetics Computer Group.
[2]Consensus sequences were taken from Kabat et al., supra.
[3]REI as sequenced by Palm et al., Hoppe-Seyler's Z. Physiol. Chem. 356: 167–191 (1975).

TABLE 2

Comparison of the mouse 21.6 heavy chain variable region to other heavy chain variable regions.

Mouse 21 6 V_H versus:	Percent Similarity[1]	Percent Identity
Consensus sequence for mouse V_H subgroup 2c[2]	94.3	91.1
Consensus sequence for human V_H subgroup 1[2]	78 0	65.0
Consensus sequence for human V_H subgroup 2[2]	70 5	53.3
Consensus sequence for human V_H subgroup 3[2]	67.5	52.8
Sequence of V_H from human 21/28'CL[3] (Member of human V_H subgroup 1)	76.5	64.7

[1]Percent similarity was determined using the "GAP" program of the University of Wisconsin Genetics Computer Group.
[2]Consensus sequences were taken from Kabat et al , supra.
[3]21/28'CL as sequenced by Dersimonian et al., J. Immunol. 139 2496–2501 (1987).

Example 2

Construction of Chimeric 21.6 Antibody

Chimeric light and heavy chains were constructed by linking the PCR-cloned cDNAs of mouse 21.6 V_L and V_H regions to human constant regions. The 5'- and 3'-ends of the mouse cDNA sequences were modified using specially designed PCR primers. The 5'-end PCR-primers (Table 3), which hybridize to the DNA sequences coding for the beginnings of the leader sequences, were designed to create the DNA sequences essential for efficient translation (Kozak, *J. Mol. Biol.* 196:947–950 (1987)), and to create a HindIII restriction sites for cloning into an expression vector. The 3'-end primers (Table 3), which hybridize to the DNA sequences coding for the ends of J regions, were designed to create the DNA sequences essential for splicing to the constant regions, and to create a BamHI site for cloning into an expression vector. The products of PCR amplification were digested with HindIII and BamHI, cloned into a pUC19 vector, and sequenced to confirm that no errors had occurred during PCR amplification. The adapted mouse 21.6 variable regions were then subcloned into mammalian cells expression vectors containing either the human kappa or gamma-1 constant regions (FIG. 3).

TABLE 3

PCR primers for the construction of chimeric 21.6 antibody.

A. Light chain variable region
1. Primer for reconstruction of the 5'-end (37mer) (SEQ. ID NO:18)

5' C AGA AAG CTT GCC GCC ACC ATG AGA CCG TCT ATT CAG 3'
HindIII Kozak Consensus Sequence — M R P S I Q

2. Primer for reconstruction of the 3'-end (35mer) (SEQ. ID NO:19)

5' CC GAG GAT CCA CTC ACG TTT GAT TTC CAG CTT GGT 3'
BamHI Splice donor site

B. Heavy chain variable region
1. Primer for reconstruction of the 5'-end (37mer) (SEQ ID NO:20)

5' C AGA AAG CTT GCC GCC ACC ATG AAA TGC AGC TGG GTC 3'
HindIII Kozak Consensus Sequence — M K C S W V

2. Primer for reconstruction of the 3'-end (33mer) (SEQ. ID NO:21)

TABLE 3-continued

PCR primers for the construction of chimeric 21.6 antibody

5' CC GAG GAT CCA CTC ACC TGA GGA GAC GGT GAC T 3'
BamHI Splice donor site

Example 3

Expression and Analysis of 21.6 Chimeric Antibody

The two plasmid DNAs coding for the chimeric 21.6 light and heavy chains were cotransfected into Cos cells. After two or three days, media from the Cos cells was analyzed by ELISA (1) for the production of a human IgG-like antibody and (2) for the ability of this human-like antibody to bind to L cells expressing human α4β1 integrin on their surface. FIGS. **4** and 12 show analyses of unpurified and protein-A purified samples of chimeric 21.6 antibody for binding to human α4β1 integrin, in comparison with purified mouse 21.6 antibody control. These figures show that the chimeric 21.6 antibody bound well to antigen and confirm that the correct mouse 21.6 V_L and V_H regions had been cloned.

Example 4

Modelling the Structure of the Mouse 21.6 Variable Regions

A molecular model of the V_L and V_H regions of mouse 21.6 antibody was built. The model was built on a Silicon Graphics IRIS 4D workstation running under the UNIX operating system and using the molecular modelling package QUANTA (Polygen Corp., USA). The structure of the FRs of mouse 21.6 V_L region was based on the solved structure of human Bence-Jones immunoglobulin REI (Epp et al., *Biochemistry* 14:4943–4952 (1975)). The structure of the FRs of mouse 21.6 V_H region was based on the solved structure of mouse antibody Gloop2. Identical residues in the FRs were retained; non-identical residues were substituted using the facilities within QUANTA. CDR1 and CDR2 of mouse 21.6 V_L region were identified as belonging to canonical structure groups 2 and 1, respectively (Chothia et al., supra). Since CDR1 and CDR2 of REI belong to the same canonical groups, CDR1 and CDR2 of mouse 21.6, V_L region were modelled on the structures of CDR1 and CDR2 of REI. CDR3 of mouse 21.6 V_L region did not appear to correspond to any of the canonical structure groups for CDR3s of V_L regions. A database search revealed, however, that CDR3 in mouse 21.6 V_L region was similar to CDR3 in mouse HyHEL-5 V_L region (Sheriff et al., *Proc. Natl. Acad. Sci. USA* 84:8075–8079 (1987)). Thus, the CDR3 of mouse 21.6 V_L region was modelled on the structure of CDR3 in mouse HyHEL-5 V_L region. CDR1 and CDR2 of mouse 21.6 V_H region were identified as belonging to canonical structure groups 1 and 2, respectively. CDR1 of mouse 21.6 V_H region was modelled on CDR1 of Gloop2 V_H region which closely resembles members of canonical group 1 for CDR1s of V_H regions. CDR2 of mouse 21.6 V_H region was modelled on CDR2 of mouse HyHEL-5 (Sheriff et al., supra), which is also a member of canonical group 2 for CDR2 for V_H regions. For CDR3s of V_H regions, there are no canonical structures. However, CDR3 in mouse 21.6 V_H region was similar to CDR3 in mouse R19.9 V_H region (Lascombe et al., *Proc. Natl. Acad. Sci. USA* 86:607–611 (1989)) and was modelled on this CDR3 by by removing an extra serine residue present at the apex of the CDR3 loop of mouse R19.9 V_H region and annealing and refining the gap. The model was finally subjected to steepest descents and conjugate gradients energy minimization using the CHARMM potential (Brooks et al., *J. Comp. Chem.* 4:187–217 (1983)) as implemented in QUANTA in order to relieve unfavorable atomic contacts and to optimize van der Waals and electrostatic interactions.

A view of the structural model of the mouse 21.6 variable regions is presented in FIG. **5**. The model was used to assist in refining the design of the humanized 21.6 antibody variable regions.

Example 5

Design of Reshaped Human 21.6 Variable Regions

(1) Selection of Homologous Human Antibodies for Framework Sequence

Human variable regions whose FRs showed a high percent identity to those of mouse 21.6 were identified by comparison of amino acid sequences. Tables 4 and 5 compare the mouse 21.6 variable regions to all known mouse variable regions and then to all known human variable regions. The mouse 21.6 V_L region was identified as belonging to mouse kappa V_L region subgroup 5 as defined by Kabat et al., supra. Individual mouse kappa V_L regions were identified that had as much as 93.4% identity to the mouse 21.6 kappa V_L region (38C13V'CL and PC613'CL). Mouse 21.6 V_L region was most similar to human kappa V_L regions of subgroup 1 as defined by Kabat et al., supra. Individual human kappa V_L regions were identified that had as much as 72.4% identity to the mouse 21.6 kappa V_L region. The framework regions (FRs) from one of the most similar human variable regions, REI, were used in the design of reshaped human 21.6 V_L region. Mouse 21.6 V_H region was identified as belonging to mouse V_H region subgroup 2c as defined by Kabat et al., supra. Individual mouse heavy chain variable regions were identified that have as much as 93.3% identity to the mouse 21.6 V_H region (17.2.25'CL and 87.92.6'CL). Mouse 21.6 V_H region was most similar to human V_H regions of subgroup 1 as defined by Kabat et al., supra. Individual human V_H regions were identified that had as much as 64.7% identity to the mouse 21.6 V_H region. The FRs from one of the most similar human variable regions, 21/28'CL, was used in the design of reshaped human 21.6 V_H region.

(2) Substitution of Amino Acids in Framework Regions

(a) Light Chain

The next step in the design process for the reshaped human 21.6 V_L region was to join the CDRs from mouse 21.6 V_L region to the FRs from human REI (Palm et al., supra). In the first version of reshaped human 21.6 V_L region (La), seven changes were made in the human FRs (Table 4, FIG. **6**).

At positions 104, 105, and 107 in FR4, amino acids from REI were substituted with more typical human J region amino acids from another human kappa light chain (Riechmann et al., *Nature* 332:323–327 (1988)).

At position 45 in FR2, the lysine normally present in REI was changed to an arginine as found at that position in mouse 21.6 V_L region. The amino acid residue at this position was thought to be important in the supporting the CDR2 loop of the mouse 21.6 V_L region.

At position 49 in FR2, the tyrosine normally present in REI was changed to an histidine as found at that position in mouse 21.6 V_L region. The histidine at this position in mouse 21.6 V_L region was observed in the model to be located in the middle of the binding site and could possibly make direct contact with antigen during antibody-antigen binding.

At position 58 in FR3, the valine normally present in REI was changed to an isoleucine as found at that position in mouse 21.6 V_L region. The amino acid residue at this

position was thought to be important in the supporting the CDR2 loop of the mouse 21.6 V_L region.

At position 69 in FR3, the threonine normally present in REI was changed to an arginine as found at that position in mouse 21.6 V_L region. The arginine at this position in mouse 21.6 V_L region was observed in the model to be located adjacent to the CDR1 loop of mouse 21.6 V_L region and could possibly make direct contact with the antigen during antibody-antigen binding.

A second version of reshaped human 21.6 V_L region (termed Lb) was designed containing the same substitutions as above except that no change was made at position 49 in FR2 of REI. (FIG. 6).

(b) Heavy Chain

The next step in the design process for the reshaped human 21.6 V_H region was to join the CDRs from mouse 21.6 V_H region to the FRs from 21/28'CL (Dersimonian et al., *J. Immunol.* 139:2496–2501 (1987)). In the first version of reshaped human 21.6 V. region (Ha), five changes were made in the human framework regions (Table 5, FIG. 7). The five changes in the human FRs were at positions 27, 28, 29, 30, and 71.

At positions 27, 28, 29, and 30 in FR1, the amino acids present in human 21/28'CL were changed to the amino acids found at those positions in mouse 21.6 V_H region. Although these positions are designated as being within FR1 (Kabat et al., supra), positions 26 to 30 are part of the structural loop that forms the CDR1 loop of the V_H region. It is likely, therefore, that the amino acids at these positions are directly involved in binding to antigen. Indeed, positions 27 to 30 are part of the canonical structure for CDR1 of the V_H region as defined by Chothia et al., supra.

At position 71 in FR3, the arginine present in human 21/28'CL was changed to a alanine as found at that position in mouse 21.6 V_H region. Position 71 is part of the canonical structure for CDR2 of the V_H region as defined by Chothia et al., supra. From the model of the mouse 21.6 variable regions, it appears that the alanine at position 71 is important in supporting the CDR2 loop of the V_H region. A substitution of an arginine for an alanine at this position would very probably disrupt the placing of the CDR2 loop.

A second version (Hb) of reshaped human 21.6 V_H region contains the five changes described above for version Ha were made plus one additional change in FR2.

At position 44 in FR2, the arginine present in human 21/28'CL was changed to a glycine as found at that position in mouse 21.6 V_H region. Based on published information on the packing Of V_L–V_H regions and on the model of the mouse 21.6 variable regions, it was thought that the amino acid residue at position 44 might be important in the packing of the V_L–V_H regions (Chothia et al., supra) (FIG. 5).

Reshaped human 21.6 V. region version Hc was designed to make the CDR3 loop look more similar to human VCAM-1. Both mouse 21.6 antibody and human VCAM-1 bind to the $\alpha 4\beta 1$ integrin. The CDR3 loop of the V_H region of antibodies is the most diverse of the six CDR loops and is generally the most important single component of the antibody in antibody-antigen interactions (Chothia et al., supra; Hoogenboom & Winter, *J. Mol. Biol.* 227:381–388 (1992); Barbas et al., *Proc. Natl. Acad. Sci. USA* 89:4457–4461 (1992)). Some sequence similarity was identified between the CDR3 of mouse 21.6 V_H region and amino acids 86 to 94 of human VCAM-1, particularly, between the YGN (Tyrosine-Glycine-Asparagine) sequence in the CDR3 loop and the FGN (Phenylalanine-Glycine-Asparagine) sequence in VCAM-1. These sequences are thought to be related to the RGD (Arginine-Glycine-Aspartic acid) sequences important in various cell adhesion events (Main et al., *Cell* 71:671–678 (1992)). Therefore, at position 98 in CDR3, the tyrosine present in mouse 21.6 V_H region was changed to a phenylalanine as found in the sequence of human VCAM-1.

Possible substitution at position 36 in FR2 was also considered. The mouse 21.6 VH chain contains an unusual cysteine residue at position 36 in FR2. This position in FR2 is usually a tryptophan in related mouse and human sequences (Table 5). Although cysteine residues are often important for conformation of an antibody, the model of the mouse 21.6 variable regions did not indicate that this cysteine residue was involved either directly or indirectly with antigen binding so the tryptophan present in FR2 of human 21/28'CL V_H region was left unsubstituted in all three versions of humanized 21.6 antibody.

Example 6

Construction of Reshaped Human 21.6 Antibodies

The first version of reshaped human 21.6 V_L region (resh21.6VLa) was constructed from overlapping PCR fragments essentially as described by Daugherty et al., *Nucleic Acids Res.* 19:2471–2476 (1991). (See FIG. 8). The mouse 21.6 V_L region, adapted as described in Example 2 and inserted into pUC19, was used as a template. Four pairs of primers, APCR1-vla1, vla2-vla3, vla4-vla5, and vla6-vla7 were synthesized (Table 6 and FIG. 8). Adjacent pairs overlapped by at least 21 bases. The APCR1 primer is complementary to the pUC19 vector. The appropriate primer pairs (0.2 μmoles) were combined with 10 ng of template DNA, and 1 unit of AmpliTaq DNA polymerase (Perkin Elmer Cetus) in 50 μl of PCR buffer containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 200 μM dNTPs, and 1.5 mM $MgCl_2$. Each reaction was carried out for 25 cycles. After an initial melt at 94° for 5 min, the reactions were cycled at 94° C. for 1 min, 55° C. for 1 min, and 72° C. for 2 min, and finally incubated at 72° C. for a further 10 min. The ramp time between the primer-annealing and extension steps was 2.5 min. The products of the four reactions (A, B, C, and D) from the first round of PCR reactions were phenol-extracted and ethanol-precipitated.

TABLE 6

PCR primers for the construction of reshaped human 21.6 variable regions.

A. Light chain variable region

1. Primers for the synthesis of version "a"

21.6VLa1 (39mer) (SEQ ID NO:22):

5' GAT GGT GAC TCT ATC TCC TAC AGA TGC AGA CAG TGA GGA 3'

21.6VLa2 (32mer) (SEQ. ID NO:23):

5' CTG TAG GAG ATA GAG TCA CCA TCA CTT GCA AG 3'

21 6VLa3 (39mer) (SEQ ID NO:24):

TABLE 6-continued

PCR primers for the construction of reshaped human 21.6 variable regions.

5' AGG AGC TTT TCC AGG TGT CTG TTG GTA CCA AGC CAT ATA 3'
21 6VLa4 (41mer) (SEQ. ID NO:25):
5' ACC AAC AGA CAC CTG GAA AAG CTC CTA GGC TGC TCA TAC AT 3'
21 6VLa5 (40mer) (SEQ. ID NO:26):
5' GCA GGC TGC TGA TGG TGA AAG TAT AAT CTC TCC CAG ACC C 3'
21 6VLa6 (42mer) (SEQ ID NO:27):
5' ACT TTC ACC ATC AGC AGC CTG CAG CCT GAA GAT ATT GCA ACT 3'
21 6VLa7 (59mer) (SEQ. ID NO:28):
5' CCG AGG ATC CAC TCA CGT TTG ATT TCC ACC TTG GTG CCT TGA CCG AAC GTC CAC AGA TT 3'
2. Primers for the synthesis of version "b"
21.6VLb1 (33mer) (SEQ. ID NO:29): changes H-49 to Y-49
5' GGA AAA GCT CCT AGG CTG CTC ATA TAT TAC ACA 3'
21.6VLb2 (38mer (SEQ. ID NO:30)): changes ACC-101 to ACA-101 to destroy an StyI site
5' CCG AGG ATC CAC TCA CGT TTG ATT TCC ACC TTT GTG CC 3'
B. Heavy chain variable region
1. Primers for the synthesis of version "a"
21 6VHa1 (51mer) (SEQ. ID NO:31):
5' AAC CCA GTG TAT ATA GGT GTC TTT AAT GTT GAA ACC GCT AGC TTT ACA GCT
21.6VHa2 (67mer) (SEQ. ID NO:32):
5' AAA GAC ACC TAT ATA CAC TGG GTT AGA CAG GCC CCT GGC CAA AGG CTG GAG TGG ATG GGA AGG ATT G 3'
21.6VHa3 (26mer) (SEQ. ID NO:33):
5' GAC CCG GCC CTG GAA CTT CGG GTC AT 3'
21.6VHa4 (66mer) (SEQ. ID NO:34):
5' GAC CCG AAG TTC CAG GGC CGG GTC ACC ATC ACC GCA GAC ACC TCT GCC AGC ACC GCC TAC ATG GAA 3'
21.6VHa5 (64mer) (SEQ. ID NO:35):
5' CCA TAG CAT AGA CCC CGT AGT TAC CAT AAT ATC CCT CTC TGG CGC AGT AGT AGA CTG CAG TGT C 3'
21.6VHa6 (63mer) (SEQ. ID NO:36).
5' GGT AAC TAC GGG GTC TAT GCT ATG GAC TAC TGG GGT CAA GGA ACC CTT GTC ACC GTC TCC TCA 3'
2. Primer for the synthesis of version "b"
21.6VHb (37mer) (SEQ. ID NO:37): changes R-44 to G-44
5' CCA GGG CCG GGTCAC CAT CAC CAG AGA CAC CTC TGC C 3'
3. Primer for the synthesis of version "c"
21.6VHc (27mer) (SEQ. ID NO:38): changes Y-98 to F-98
5' CAG GCC CCT GGC CAA GGG CTG GAG TGG 3'
C. Both light and heavy chain variable regions
Primers hybridizing to the flanking pUC19 vector DNA
APCR1 (17mer (SEQ. ID NO:39), sense primer)
5' TAC GCA AAC CGC CTC TC 3'
APCR4 (18mer (SEQ. ID NO:40), anti-sense primer)
5' GAG TGC ACC ATA TGC GGT 3'

PCR products A and B, and C and D were joined in a second round of PCR reactions. PCR products A and B, and C and D, (50 ng of each) were added to 50 μl PCR reactions (as described above) and amplified through 20 cycles as described above, except that the annealing temperature was raised to 60° C. The products of these reactions were termed E and F. The pairs of PCR primers used were APCR1-vla3 and vla4-vla7, respectively. PCR products E and F were phenol-extracted and ethanol-precipitated and then assembled in a third round of PCR reactions by their own complementarity in a two step-PCR reaction similar to that described above using APCR1 and vla7 as the terminal primers. The fully assembled fragment representing the entire reshaped human 21.6 V_L region including a leader sequence was digested with HindIII and BamHI and cloned into pUC19 for sequencing. A clone having the correct sequence was designated resh21.6VLa

The second version of a reshaped human 21.6 V_L region (Lb) was constructed using PCR primers to make minor modifications in the first version of reshaped human 21.6 V_L region (La) by the method of Kamman et al., *Nucl. Acids Res.* 17:5404 (1989). Two sets of primers were synthesized (Table 6). Each PCR reaction was essentially carried out under the same conditions as described above. In a first PCR reaction, mutagenic primer 21.6VLb2 was used to destroy a StyI site (Thr-ACC-97 to Thr-ACA-97) to yield resh21.6VLa2. Then, in a second PCR reaction, mutagenic primer 21.6VLb1 (His-49 to Tyr-49) was used with pUC-resh21.6VLa2 as template DNA. The PCR product was cut with StyI and BamHI and subcloned into pUC-resh21.6VLa2, cleaved with the same restriction enzymes. A clone with the correct sequence was designated pUC-resh21.6VLb.

Version "a" of a reshaped human 21.6 V_H region was constructed using the same PCR methods as described for the construction of version "a" of reshaped human 21.6 V_L region (Table 6 and FIG. 9). The HindIII-BamHI DNA fragments coding for version "g" of reshaped human 425 V_H region (Kettleborough et al., supra) and version "b" of reshaped human AUK12-20 V_H region were subcloned into pUC19 vectors yielding pUC-resh425g and pUC-reshAUK12-20b, respectively. (Version "b" of AUK12-20, was derived by PCR mutagenesis of a fragment V_Ha425 described by Kettleborough et al., supra, and encodes the amino acid sequence (SEQ. ID NO:41):

QVQLVQSGAEVKKPGASVKVSCKASGYSFT SYYIH
WVRQAPGQGLEWVG YIDPFNGGTSYNQKFKG

KVTMTVDTSTNTAYMELSSLRSEDTAVYYCAR GGN-RFAY WGQGTLVTVSS (spaces separate FR and CDR regions)).

Plasmid pUC-resh425g and pUC-reshAUK12-20b, as well as the pUC vector containing the mouse 21.6 V_H region as modified for use in the construction of the chimeric 21.6 heavy chain (pUC-chim21.6V_H), were used as template DNAs in the subsequent PCR reactions. PCR primers were designed and synthesized for the construction of version "a" of reshaped human 21.6 V_H region (Table 6). PCR product A (FIG. 9) was obtained using pUC-reshAUK12-20b as DNA template and APCR1-vha1 as the PCR primer pair. PCR products B and D were obtained using pUC-chim21.6V_H as DNA template and vha2-vha3 and vha6-APCR4 as PCR primer pairs, respectively. Finally, PCR product C was obtained using pUC-resh425g as DNA template and vla4-vla5 as the PCR primer pair. The final PCR product was subcloned into pUC19 as an HindIII-BamHI fragment for DNA sequencing. A clone with the correct DNA sequence was designated pUC-resh21.6VHa. The DNA and amino acid sequences of the first version of the reshaped 21.6 variable region are shown in FIG. 10.

The remaining versions of reshaped human 21.6 V_H region were constructed essentially as described above for the construction of version "b" of reshaped human 21.6 V_L region. Two sets of primers were synthesized (Table 6). For the second (Hb) and third (Hc) versions, mutagenic primers 21.6VHb (Arg-44 to Gly-44) and 21.6VHc (Tyr-98 to Phe-98), respectively, were used in PCR reactions with pUC-resh21.6VHa as the template DNA. The PCR products VHb and VHc were cut with restriction enzymes and subcloned into pUC vector pUC-resh21.6VHa as MscI-BamHI and PstI-BamHI fragments, respectively, to yield pUC-resh21.6VHb and pUC-resh21.6VHc.

The first version of a reshaped human 21.6 V_H region (Ha) was constructed in a similar manner to that used for the construction of the first version of reshaped human 21.6 V_L region (La). In this case, however, PCR primers were used with three different template DNAs, mouse 21.6 V_H region as already adapted for expression of chimeric 21.6 heavy chain, humanized 425 V_H region version "g" (Kettleborough et al., supra), and humanized AUK12-20 version "b" V_H region (Table 6, FIG. 9). The DNA and amino acid sequences of the first version of the humanized 21.6 heavy chain variable region are shown in FIG. 11. The second and third versions of a humanized 21.6 V_H region (Hb and Hc) were constructed using PCR primers to make minor modifications in the first version of humanized 21.6 V_H region (Ha) (Table 6).

Example 7

Expression and Analysis of Humanized Antibodies

1. Linkage of Variable Regions to Constant Regions in Expression Vectors

The DNA fragments coding for the chimeric and reshaped 21.6 V_L and V_H regions were subcloned into HCMV vectors designed to express either human kappa light chains or human gamma-1 heavy chains in mammalian cells (see FIG. 3) and Maeda et al., Hum. Antibod. Hybridomas 2:124–134 (1991). Both vectors contain the human cytomegalovirus (HCMV) promoter and enhancer for high level transcription of the immunoglobulin light and heavy chains. The light chain expression vector is exactly as described in Maeda et al., supra, and contains genomic DNA coding for the human kappa constant region (Rabbitts et al., Curr. Top. *Microbiol. Immunol.* 113:166–171 (1984)). The heavy chain expression vector is essentially as described in Maeda et al., supra, with the exception that the genomic DNA coding for the human gamma-1 constant region was replaced with a cDNA. cDNA coding for human gamma-1 constant region was cloned from a human cell line that secreted a human gamma-1 antibody by PCR. For convenient subcloning into the expression vector, BamHI sites were created at each end of the cDNA. In addition, a splice acceptor site and a 65 bp intron sequence were created at the 5'-end of the cDNA sequence. The BamHI fragment (1176 bp) containing the human gamma-1 cDNA splice acceptor site and intron sequence was substituted for the BamHI fragment (approximately 2.0 kb) in the existing heavy chain vector (Maeda et al., supra). The BamHI site to the 3'-side of the human gamma-1 constant region was then removed with Klenow polymerase.

2. Transfection of Expression Vectors

Expression vectors were introduced into Cos cells by electroporation using the Gene Pulsar apparatus (BioRad). DNA (10 μg of each vector) was added to a 0.8 ml aliquot of 1×10^7 cells/ml in PBS. A pulse was delivered at 1,900 volts, 25 μF capacitance. After a 10 min recovery period at ambient temperature, the electroporated cells were added to 8 ml of DMEM (GIBCO) containing 5% heat-inactivated gamma globulin-free fetal calf serum. After 72 h incubation, the medium was collected, centrifuged to remove cellular debris, and stored under sterile conditions at 4° C. for short periods of time, or at −20° C. for longer periods.

3. Purification of Humanized Antibodies

Supernatants from Cos cell transfections were pooled and purified on immobilized Protein A (ImmunoPure IgG Purification Kit, Pierce). The supernatant was sterilized by filtration through a 0.22 μm filter. After mixing with an equal volume of ImmunoPure IgG binding buffer (pH 8.0), the diluted sample was applied to a 1 ml protein A column and allowed to flow completely into the gel. After washing with 15 ml of ImmunoPure IgG binding buffer, the bound antibody was eluted with 5 ml of ImmunoPure IgG elution buffer (pH 2.8), and 1 ml fractions were collected. The pH of the first and second fractions was approximately 8.0. The pH of the third fraction was adjusted to physiological pH by the addition of 100 μl of ImmunoPure binding buffer. The five 1 ml fractions containing the Protein A-purified antibody were then assayed by ELISA to determine the amount of human IgG antibody present in each fraction. Antibody was detected using goat alkaline phosphate-conjugated anti-human IgG (whole molecule, Sigma).

4. Measurement of Binding Affinity

The binding of reshaped human 21.6 antibodies to $\alpha4\beta1$ integrin was assayed by ELISA in comparison with mouse and chimeric antibodies. Briefly, L cells transformed to express $\alpha4\beta1$ integrin on their cell surface were plated out and grown to confluence in 96-well tissue culture plates. The samples to be tested (either crude supernatants or protein-A-purified) were serially diluted and added to each well. After incubation for 1 h on ice and very gentle washing, goat anti-mouse or anti-human (gamma-chain specific) peroxidase conjugates (Sigma) were added. After a further 1 h incubation on ice and very gentle washing, the substrate (o-phenylenediamine dihydrochloride, Sigma) was added. After incubation for 30 min at room temperature, the reaction was stopped by adding 1M H_2SO_4, and the A_{490} was measured.

Results from analyzing crude supernatants of the two versions of reshaped human 21.6 light chains (La and Lb), in combination with version Ha of reshaped human 21.6 heavy chain, indicated that the La version of reshaped human 21.6 V_L region gave slightly better binding to antigen than version Lb. The La version was therefore used in

subsequent experiments. Results from analysis of the crude supernatants of humanized 21.6 heavy chains (Ha and Hb), in combination with version La of humanized 21.6 light chain, showed no significant difference between the two versions (Ha and Hb) of reshaped human V_H regions. Version Ha was selected for use in further experiments because it contained only five changes in the human FRs compared with six changes in the human Hb.

FIG. 12 compares binding of humanized 21.6 antibody (La+Ha) with chimeric 21.6 antibody. The data indicate that the reshaped human 21.6 antibody (La +Ha) bound to antigen as well as, and perhaps slightly better than, the chimeric 21.6 antibody. The chimeric 21.6 antibody is expected to be equivalent to mouse 21.6 antibody in its antigen binding characteristics because it contains the intact mouse 21.6 variable regions. The reshaped human 21.6 antibody (La+Ha) has also been shown to block binding to human α4β1 integrin with an efficiency comparable to the original mouse 21.6 antibody and to the chimeric antibody. It is therefore concluded that reshaped human 21.6 antibody (La+Ha) has a specific binding affinity essentially equal to that of mouse 21.6 antibody. Moreover, because only minor modifications in the human FRs were necessary to recreate the antigen binding site of mouse 21.6 antibody within human variable regions, the reshaped human 21.6 antibody is predicted to behave like an authentic human antibody.

Reshaped human 21.6 antibody containing version La of the reshaped human 21.6 V_L region and version Hc of the reshaped human 21.6 V_H region was also tested for binding to L cells expressing human α4β1 integrin on their surface in parallel with chimeric 21.6 antibody. The results indicate that reshaped human 21.6 antibody (La+Hc) binds well to antigen. The alteration in the CDR3 of the V_H region did not impair binding to antigen. Indeed, there is some indication that the alteration in the CDR3 may have slightly improved binding to antigen (FIG. 12). Conceivably, the improvement may be more pronounced in a functional blocking assay.

Example 8

Blocking Properties of Mu 21.6 Antibody

Mu 21.6 was compared with another antibody against α_4 integrin called L25. L25 is commercially available from Becton Dickinson, and has been reported in the literature to be a good inhibitor of $\alpha_4\beta_1$ integrin adhesive function. As shown in FIG. 13 (Panel A), both Mu 21.6 and L25 completely inhibited $\alpha_4\beta_1$ integrin-dependent adhesion of human monocytic cells to purified VCAM-1 in the absence of Mn^{+2}. However, in the presence of Mn^{+2} (1 mM) (one of several activators of $\alpha_4\beta_1$ integrin) L25 was no longer an effective inhibitor. Similar results were observed when $\alpha_4\beta_1$ integrin was activated by other stimuli. The capacity to block activated α4β1 integrin is likely to be of value in treating inflammatory diseases such as multiple sclerosis.

As a further comparison between mu 21.6 and L25, the capacity of antibody to inhibit human T cell adhesion to increasing amounts of VCAM-1 was determined. In this experiment, increasing amounts of VCAM-1 were coated onto plastic wells of a 96 well assay plate, and the ability of the human T cell line, Jurkat (which expresses high levels of $\alpha_4\beta_1$ integrin), to bind to the coated wells was measured. Values on the Y-axis represent the percentage of Jurkat cells originally added to each well that remained bound after washing the well four times (FIG. 13 (Panel B)). This experiment demonstrates that L25 is a good inhibitor of cell adhesion when low levels of VCAM-1 are encountered, but becomes completely ineffective at higher levels of VCAM-1. Mu 21.6, on the other hand, inhibits cell adhesion completely, regardless of the amount of VCAM-1 present. The capacity to block at high concentrations of VCAM-1 is desirable for therapeutic applications because of upregulation of VCAM-1 at sites of inflammation.

Example 9

Efficacy of Humanized 21.6 Antibody in An Animal Model

This example establishes the efficacy of humanized 21.6 antibody in prophylactic and therapeutic treatment of EAE in an animal model simulating multiple sclerosis in humans.

(a) Methods

(1) Induction of EAE

The brain and spinal cord were removed from each of five guinea pigs euthanized by CO_2 narcosis. The tissue was kept in PBS on wet ice until it was weighed and homogenized at a concentration of 1 gram of tissue per ml PBS. The tissue was completely homogenized using an electric hand-held homogenizer and subsequently mixed with an equal volume of Freund's complete adjuvant (FCA). FCA was made by adding 100 mg of *mycobacterium tuberculosis* H37 RA (DIFCO, 3114–33–8) to 10 ml of Freund's incomplete adjuvant (Sigma, F-5506). The mixture was emulsified into the consistency of mayonnaise by passing the solution between two syringes connected by a two way stopcock. Each guinea pig was immunized with 600 μl emulsion divided between three sites of administration.

(2) Scoring animals for disease symptoms

The disease symptoms were assessed by prompting each animal to walk and assigning the animal a score by the following commonly accepted criteria:

0	No disease
1	Hind limb weakness
2	Complete hind limb paralysis
3	Complete hind limb and some forelimb paralysis
4	Moribund or dead

(3) Serum and tissue collection

Samples were collected by cardiac puncture from methoxyflurane-anesthetized guinea pigs. About 300–400 μl of blood were collected and placed in microtainer serum separator and allowed to clot for between 20–30 min at room temperature. The tube was then spun for 5 min at room temperature. The serum was drawn off into Eppendorf tubes and stored at −20° C. for subsequent analysis of antibody titers by fluorescence activated cell sorting (FACS).

For hematological analysis, blood was collected into ethylenediaminetetraacetic acid-coated microtainer tubes. A 100 μl aliquot was aspirated into an acridine-coated hematocrit tube. The tube was capped and the blood was mixed with acridine orange by gently inverting the tube 15 times. A float was put into the hematocrit tube and the sample was centrifuged for 5 minutes. The hematocrit tube was placed into a precalibrated Idexx QBC Vet Autoreader designed for quantitative buffey coat analysis. Values were read under the horse calibration system and adjusted to guinea pig equivalents using a predetermined conversion factor.

At the end of the experiment, the guinea pigs were killed by CO_2 narcosis and the brains and spinal cords removed. Half of the brain and spinal cord from every guinea pig was snap frozen in 2-methyl butane on dry ice (−20° to −40° C.). This tissue was cut and immunostained with a pan macrophage marker (Serotec MCA-518) and a T-lymphocyte marker (Serotec MCA-751) using the avidin-biotin linking peroxidase assay (Vector Laboratories, Inc., Burlingame, Calif.) and diaminobenzidine as a chromagen. The tissue was scored for cellular infiltration according to the following scoring system:

0	No infiltrating cells.
0.5	Very little staining; may be artifactual; usually associated with vessels.
1	Staining of a few cells (less than 15) usually near a vessel.
2	Staining of many cells (20–50), usually radiating out from a vessel
3	Staining of many cells (>50) scattered throughout the tissue; many cuffed vessels

(b) Prophylactic Treatment

This experiment was designed to evaluate the efficacy of humanized 21.6 antibody in delaying the onset of clinical symptoms. Previous data have demonstrated that leukocyte influx into the brain and spinal cord of EAE guinea pigs typically starts between day 7 and day 8. Therefore, antibodies were administered on day 7 and on day 10 post-immunization. To compare mouse and humanized 21.6 antibody, equivalent doses of each of the antibodies (3.0, 0.30 and 0.03 mg/kg) were administered. Preliminary pharmacokinetic studies revealed that saturating blood levels of mouse 21.6 antibody were attained within 24 hours after subcutaneous administration, and remained elevated up to 48 hours.

On day 11, 24 hours after the second dose of antibody, blood samples were drawn from three randomly selected animals in each group. For each treatment group a mean for the number of days for each guinea pig to reach a clinical score of 1 was calculated (Table 7). The mean value for the PBS-treated group in this experiment was 11 days post-immunization (which is typical of previous results). Treatment with the highest dose of humanized and mouse antibody resulted in a significant delay of disease by 4.6 (p=0.000) and 3 (p=0.007) days, respectively. The lower doses of antibody had no effect on the course of disease.

TABLE 7

Effect of mouse or humanized 21.6 antibody on time post immunization to reach a clinical score of 1

GROUP mg/kg	1 0.03 M#	2 3.0 H@	3 3.0 H	4 3.0 M	5 0.03 H	6 PBS	7 0.3 M
	8	9	13	10	8	9	9
	9	10	15	12	10	9	9
	9	10	15	14	10	11	11
	9	11	16	14	11	11	12
	11	11	16	14	12	11	12
	12	11	16	15	12	12	13
	12	12	17	15	12	12	13
		13	17	18	12	13	
Mean ± SD	10.0 ± 1.6	10.9 ± 1.2	**15.6 ± 1.3	*14.0 ± 2.3	10.9 ± 1.5	11.0 ± 1.4	11.6 ± 1.4

@H denotes humanized antibody; #M denotes mouse. **p = 0.000 and p = 0.007, as compared to PBS.

Daily body weights of the guinea pig reflected a similar effect of the high doses of humanized and mouse antibody. (FIG. 14). Animals in these treatment groups steadily gained weight. Guinea pigs in all other treatment groups lost weight starting from just before the day of onset of disease.

Serum titers of antibody were measured in three randomly selected animals from each group by cardiac puncture on day 11, roughly 24 hr after the second treatment. Efficacy of the antibodies to delay disease correlated tightly with serum levels. About 20 μg/ml serum antibody was present in the circulation of all animals injected with the highest dose of both humanized and mouse antibodies. This concentration is of the same order of magnitude as the concentration of 21.6 antibody required to saturate VLA-4 sites in vitro. In contrast, animals from all other groups had little to no detectable serum antibody.

(c) Reversal of On-going Disease

About 60 guinea pigs were immunized and allowed to develop clinical symptoms of EAE. On day 13, all guinea pigs that attained a clinical score of 1 were randomly assigned to a treatment group. FIG. 15 shows that animals treated with 3 mg/kg humanized antibody began to recover hind limb function within 48 hr of treatment. On Days 17 and 18, one and two days after the second dose, all eight animals were disease free. ANOVA of the area under the curve values for each treatment group revealed that only the 3 mg/kg humanized antibody treated group value was statistically lower than the PBS control group (p=0.042). These animals progressively gained weight within 24 hrs after the first administration until the experiment was terminated on Day 19 (FIG. 16).

Antibody serum titers were measured by FACS analysis on samples taken 24 hrs after the first injection (Day 14) and at sacrifice (Day 19). Treatment with mouse 21.6 antibody resulted in slightly lower serum antibody titers than treatment with humanized 21.6 antibody (9.1 vs. 12.6 μg/ml). This difference became more profound on Day 19, three days after the second administration, when there was very little detectable serum mouse antibody, while the levels of humanized antibody on Day 19 had dropped below saturating but were still measurable (6.1 μg/ml). These data demonstrate a correlation between plasma levels of antibody and physiologic efficacy and suggest that the effective circulating antibody level is in the range of 10–20 μg/ml in the guinea pig.

Leukocyte infiltration onto brain and spinal cord was evaluated in tissue from animals killed on Day 19. Table 8 shows significant differences in the degree of infiltration as a function of antibody treatment. The reduction in T cell infiltration into brain and spinal cord and macrophage infiltration into spinal cord was significant after treatment with 3 mg/kg. Lower doses tended to reduce infiltration, but did not reach significance. There was no significant difference in cellular infiltrate of macrophages into the spinal cord at any dose. Since the immunohistochemical technique used to evaluate macrophages does not distinguish resident from invading cells, the lack of effect on macrophages likely represents the sustained presence of resident macrophages and microglia.

The reduction in T-cells and monocytes in brain tissue by administration of the antibody after establishment of the disease suggests that cell trafficking is not a cumulative process, but a dynamic movement of cells into and out of CNS tissue. Importantly, the data suggest that interruption of the entry of leukocytes into parenchymal tissue allows the CNS to rid itself of the invading pathological element.

TABLE 8

Significant differences in T-cell and macrophage infiltration into brain and spinal cord on Day 129

GROUP PBS	BRAIN T-CELLS	BRAIN MACRO-PHAGES	SPINAL CORD T-CELLS	SPINAL CORD MACRO-PHAGES
3 mg/kg @ H	p = 0.001	p = 0.005	p = 0.007	NS
3 mg/kg # M	p = 0.001	p = 0.005	p = 0.008	NS

TABLE 8-continued

Significant differences in T-cell and macrophage infiltration into brain and spinal cord on Day 129

GROUP PBS	BRAIN T-CELLS	BRAIN MACRO-PHAGES	SPINAL CORD T-CELLS	SPINAL CORD MACRO-PHAGES
1 mg/kg H	NS	NS	NS	NS
0.3 mg/kg H	NS	NS	NS	NS

NS = not significant.

Hematology data revealed that treatment with mouse or humanized 21.6 antibody caused no difference in whole white blood cell counts, mononuclear and granulocyte number or in red blood cell count. The high dose of mouse or humanized antibody resulted in a significant increase in platelet counts as compared to PBS treated animals (Table 9). In normal guinea pig platelet counts are 755±103 cells/ml, about double that of PBS-treated EAE animals. Thus, treatment with doses of mouse and humanized antibody that effectively reversed disease, also restored platelet count to normal.

TABLE 9

Effect of antibody treatment on platelet count in EAE animals

TREATMENT	PLATELETS x 10^{-6} CELLS/ML
++Non EAE guinea pigs	755 ± 103 (9)
PBS	373.7 ± 167.5 (7)
3 mg/kg @H	622.2 ± 97.0 (6)**
3 mg/kg #M	587.5 ± 57.8 (6)
1 mg/kg H	578.3 ± 123.2 (6)
0.3 mg/kg H	492.5 ± 168.6 (6)

++Platelet counts in non-EAE guinea pigs were determined in a separate experiment.
*p = 0.05 vs PBS.

In conclusion, both humanized and mouse 21.6 antibodies are effective in delaying and reversing clinical symptoms in an animal model simulating multiple sclerosis in humans. The humanized antibody is more effective than the same dosage of mouse antibody in reversing symptoms.

Although the foregoing invention has been described in detail for purposes of clarity of understanding, it will be obvious that certain modifications may be practiced within the scope of the appended claims. All publications and patent documents cited above are hereby incorporated by reference in their entirety for all purposes to the same extent as if each were so individually denoted.

TABLE 4

Alignment of amino acid sequences leading to the design of reshaped human 21.6 light chain variable regions.

Kabat #		FR or CDR	mouse 21.6	mouse kappa 5 (SEQ. ID NO:42)	human kappa 1 (SEQ. ID NO:43)	human REI	RH V_L 21.6	Comment
1	1	FR1	D	D	D	D	D	
2	2	|	I	I	I	I		
3	3	|	Q	Q	Q	Q	Q	
4	4	|	M	M	M	M	M	
5	5	|	T	T	T	T	T	
6	6	|	Q	Q	Q	Q	Q	
7	7	|	S	S	S	S	S	
8	8	|	P	P	P	P	P	
9	9	|	S	S	S	S	S	
10	10	|	S	S	S	S	S	
11	11	|	L	L	L	L	L	
12	12	|	S	S	S	S	S	
13	13	|	A	A	A	A	A	
14	14	|	S	S	S	S	S	
15	15	|	L	L	V	V	V	
16	16	|	G	G	G	G	G	
17	17	|	G	D	D	D	D	
18	18	|	K	R	R	R	R	
19	19	|	V	V	V	V	V	
20	20	|	T	T	T	T	T	
21	21	|	I	I	I	I	I	
22	22	|	T	T	T	T	T	
23	23	FR1	C	C	C	C	C	
24	24	CDR1	K	R	R	Q	K	
25	25	|	T	A	A	A	T*	
26	26	|	S	S	S	S	S*	
27	27	|	Q	Q	Q	Q	Q*	
27A		|	—	D	S	—	—	
27B		|	—	—	L	—	—	
27C		|	—	—	V	—	—	
27D		|	—	—	X	—		
27E		|	—	—	X	—	—	
27F		|	—	—	—	—	—	
28	28	|	D	D	S	D	D*	
29	29	|	I	I	I	I	I*	
30	30	|	N	S	S	I	N*	
31	31	|	K	N	N	K	K*	
32	32	|	Y	Y	Y	Y	Y*	

TABLE 4-continued

Alignment of amino acid sequences leading to the design of reshaped human 21.6 light chain variable regions

Kabat	#	FR or CDR	mouse 21 6	mouse kappa 5 (SEQ. ID NO:42)	human kappa 1 (SEQ. ID NO:43)	human REI	RH V_L 21.6	Comment
33	33	|	M	L	L	L	M*	
34	34	CDR1	A	N	A	N	A	
35	35	FR2	W	W	W	W	W	
36	36	|	Y	Y	Y	Y	Y	
37	37	|	Q	Q	Q	Q	Q	
38	38	|	H	Q	Q	Q	Q	
39	39	|	K	K	K	T	T	K in CAMPATH-1H
40	40	|	P	P	P	P	P	
41	41	|	G	G	G	G	G	
42	42	|	K	G	K	K	K	
43	43	|	R	S	A	A	A	consider R in other versions
44	44	|	P	P	P	P	P	
45	45	|	R	K	K	K	R	supports L2 loop, consider K in other versions
46	46	|	L	L	L	L	L	
47	47	|	L	L	L	L	L	
48	48	|	I	I	I	I	I*	
49	49	|	H	Y	Y	Y	H	in middle of binding site, potential to interact with antigen, consider Y in other versions
50	50	CDR2	Y	Y	A	E	Y*	
51	51	|	T	A	A	A	T*	
52	52	|	S	S	S	S	S*	
53	53	|	A	R	S	N	A	
54	54	|	L	L	L	L	L	
55	55	|	Q	H	E	Q	Q	
56	56	CDR2	P	S	S	A	P	
57	57	FR3	G	G	G	G	G	
58	58	|	I	V	V	V	I	maybe supporting L2, consider V in other versions
59	59	|	P	P	P	P	P	
60	60	|	S	S	S	S	S	
61	61	|	R	R	R	R	R	
62	62	|	F	F	F	F	F	
63	63	|	S	S	S	S	S	
64	64	|	G	G	G	G	G*	
65	65	|	S	S	S	S	S	
66	66	|	G	G	G	G	G	
67	67	|	S	S	S	S	S	
68	68	|	G	G	G	G	G	
69	69	|	R	T	T	T	R	adjacent to L1, on the surface near the binding site
70	70	|	D	D	D	D	D	
71	71	|	Y	Y	F	Y	Y*	F in CAMPATH-1H
72	72	|	S	S	T	T	T	
73	73	|	F	L	L	P	F	
74	74	|	N	T	T	T	T	
75	75	|	I	I	I	I	I	
76	76	|	S	S	S	S	S	
77	77	|	N	N	S	S	S	
78	78	|	L	L	L	L	L	

TABLE 4-continued

Alignment of amino acid sequences leading to the design of reshaped human 21.6 light chain variable regions.

Kabat	#	FR or CDR	mouse 21.6	mouse kappa 5 (SEQ. ID NO:42)	human kappa 1 (SEQ. ID NO:43)	human REI	RH V_L 21.6	Comment
79	79	|	E	E	Q	Q	Q	
80	80	|	P	Q	P	P	P	
81	81	|	E	E	E	E	E	
82	82	|	D	D	D	D	D	
83	83	|	I	I	F	I	I	
84	84	|	A	A	A	A	A	
85	85	|	T	T	T	T	T	
86	86	|	Y	Y	Y	Y	Y	
87	87	|	Y	F	Y	Y	Y	
88	88	FR3	C	C	C	C	C	
89	89	CDR3	L	Q	Q	Q	L	
90	90	|	Q	Q	Q	Q	Q*	
91	91	|	Y	G	Y	Y	Y*	
92	92	|	D	N	N	Q	D*	
93	93	|	N	T	S	S	N*	
94	94	|	L	L	L	L	L*	
95	95	|	—	P	P	P	—	
95A		|	—	P	E	—	—	
95B		|	—	—	—	—	—	
95C		|	—	—	—	—	—	
95D		|	—	—	—	—	—	
95E		|	—	—	—	—	—	
95F		|	—	—	—	—	—	
96	95	|	W	R	W	Y	W*	
97	96	CDR3	T	T	T	T	T	
98	97	FR4	F	F	F	F	F	
99	98	|	G	G	G	G	G	
100	99	|	G	G	Q	Q	Q	
101	100	|	G	G	G	G	G	
102	101	|	T	T	T	T	T	
103	102	|	K	K	K	K	K	
104	103	|	L	L	V	L	V	as in CAMPATH-1H
105	104	|	E	E	E	Q	E	as in CAMPATH-1H
106	105	|	I	I	I	I	I	
106A		|	—	—	—	—		
107	106	FR4	K	K	K	T	K	as in CAMPATH-1H

Legend: (Kabat) numbering according to Kabat et al., supra; (#) sequential numbering as used in the molecular modelling; (mouse 21.6) amino acid sequence of the V_L region from mouse 21.6 antibody; (mouse kappa 5) consensus sequence of mouse kappa V_L regions from subgroup 5 (Kabat et al., supra); (human kappa 1) consensus sequence of human V_L regions from subgroup 1 (Kabat et al., supra); (human RED amino acid sequence of a human V_L region (Palm et al. (1975), supra); (RH V_L 21.6) amino acid sequence of version L1 of reshaped human 21.6 V_L region; (*) residues that are part of the canonical structures for the CDR loops (Chothia et al., supra); (underlined) residues in the human FRs where the amino acid residue was changed.

TABLE 5

Alignment of amino acid sequences leading to the design of reshaped human 21.6 heavy chain variable regions

Kabat	#	FR or CDR	mouse 21.6	mouse 2c (SEQ. ID NO:44)	human 1 (SEQ. ID NO:45)	human 21/28'CL	RH V_H 21.6	Comment
1	1	FR1	E	E	Q	Q	Q	
2	2	|	V	V	V	V	V	
3	3	|	Q	Q	Q	Q	Q	
4	4	|	L	L	L	L	L	
5	5	|	Q	Q	V	V	V	
6	6	|	Q	Q	Q	Q	Q	
7	7	|	S	S	S	S	S	

TABLE 5-continued

Alignment of amino acid sequences leading to the design of reshaped human 21.6 heavy chain variable regions.

Kabat	#	FR or CDR	mouse 21.6	mouse 2c (SEQ. ID NO:44)	human 1 (SEQ. ID NO:45)	human 21/28'CL	RH V_H 21.6	Comment
8	8	|	G	G	G	G	G	
9	9	|	A	A	A	A	A	
10	10	|	E	E	E	E	E	
11	11	|	L	L	V	V	V	
12	12	|	V	V	K	K	K	
13	13	|	K	K	K	K	K	
14	14	|	P	P	P	P	P	
15	15	|	G	G	G	G	G	
16	16	|	A	A	A	A	A	
17	17	|	S	S	S	S	S	
18	18	|	V	V	V	V	V	
19	19	|	K	K	K	K	K	
20	20	|	L	L	V	V	V	
21	21	|	S	S	S	S	S	
22	22	|	C	C	C	C	C	
23	23	|	T	T	K	K	K	
24	24	|	A	A	A	A	A	
25	25	|	S	S	S	S	S	
26	26	|	G	G	G	G	G*	
27	27	|	F	F	Y	Y	F*	H1 canonical structure, consider Y in other versions
28	28	|	N	N	T	T	N*	H1 canonical structure, on the surface
29	29	|	I	I	F	F	I*	H1 canonical structure, consider F in other versions
30	30	FR1	K	K	T	T	K*	H1 canonical structure, on the surface
31	31	CDR1	D	D	S	S	D*	
32	32	|	T	T	Y	Y	T*	
33	33	|	Y	Y	A	A	Y	
34	34	|	I	M	I	M	I*	
35	35	|	H	H	S	H	H	
35A		|	—	—	—	—	—	
35B		CDR1	—	—	—	—	—	
36	36	FR2	C	W	W	W	W	buried residue, no obvious special role for C
37	37	|	V	V	V	V	V	
38	38	|	K	K	R	R	R	
39	39	|	Q	Q	Q	Q	Q	
40	40	|	R	R	A	A	A	
41	41	|	P	P	P	P	P	
42	42	|	E	E	G	G	G	
43	43	|	Q	Q	Q	Q	Q	
44	44	|	G	G	G	R	R	$V_L - V_H$ packing, consider G in other versions
45	45	|	L	L	L	L	L	
46	46	|	E	E	E	E	E	
47	47	|	W	W	W	W	W	
48	48	|	I	I	M	M	M	
49	49	FR2	G	G	G	G	G	
50	50	CDR2	R	R	W	W	R	

TABLE 5-continued

Alignment of amino acid sequences leading to the design of reshaped human 21.6 heavy chain variable regions.

Kabat	#	FR or CDR	mouse 21.6	mouse 2c (SEQ. ID NO:44)	human 1 (SEQ. ID NO:45)	human 21/28'CL	RH V_H 21.6	Comment
51	51		I	I	I	I	I	
52	52		D	D	N	N	D	
52A	53		P	P	P	A	P*	
52B			—	—	Y	—	—	
52C			—	—	—	—	—	
53	54		A	A	G	G	A*	
54	55		N	N	N	N	N*	
55	56		G	G	G	G	G*	
56	57		Y	N	D	N	Y	
57	58		T	T	T	T	T	
58	59		K	K	N	K	K	
59	60		Y	Y	Y	Y	Y	
60	61		D	D	A	S	D	
61	62		P	P	Q	Q	P	
62	63		K	K	K	K	K	
63	64		F	F	F	F	F	
64	65		Q	Q	Q	Q	Q	
65	66	CDR2	G	G	G	G	G	
66	67	FR3	K	K	R	R	R	
67	68		A	A	V	V	V	
68	69		T	T	T	T	T	
69	70		I	I	I	I	I	
70	71		T	T	T	T	T	
71	72		A	A	A	R	A*	H2 canonical structure, supportin g H2
72	73		D	D	D	D	D	
73	74		T	T	T	T	T	
74	75		S	S	S	S	S	
75	76		S	S	T	A	A	
76	77		N	N	S	S	S	
77	78		T	T	T	T	T	
78	70		A	A	A	A	A	
79	80		Y	Y	Y	Y	Y	
80	81		L	L	M	M	M	
81	82		Q	Q	E	E	E	
82	83		L	L	L	L	L	
82A	84		S	S	S	S	S	
82B	85		S	S	S	S	S	
82C	86		L	L	L	L	L	
83	87		T	T	R	R	R	
84	88		S	S	S	S	S	
85	89		E	E	E	E	E	
86	90		D	D	D	D	D	
87	91		T	T	T	T	T	
88	92		A	A	A	A	A	
89	93		V	V	V	V	V	
90	94		Y	Y	Y	Y	Y	
91	95		F	Y	Y	Y	Y	
92	96		C	C	C	C	C	
93	97		A	A	A	A	A	
94	98	FR3	R	R	R	R		
95	99	CDR3	E	G	A	G	E	
96	100		G	Y	P	G	G	
97	101		Y	Y	G	Y	Y	
98	102		Y	Y	Y	Y	Y	
99	103		G	Y	G	G	G	
100	104		N	D	S	S	N	
100A	105		Y	S	G	G	Y	
100B	106		G	X	G	S	G	
100C	107		V	V	o	—	V	
100D	108		Y	G	C	—	Y	
100E	109		A	Y	Y	—	A	
100F	110		M	Y	R	M		
100G			—	A	0	—	—	
100H			—	M	D	—	—	
100I			—	—	Y	—	—	
100J			—	—	—	—		
100K			—	—	F	—	—	
101	111		D	D	D	N	D	

TABLE 5-continued

Alignment of amino acid sequences leading to the design of reshaped human 21.6 heavy chain variable regions.

Kabat	#	FR or CDR	mouse 21.6	mouse 2c (SEQ. ID NO:44)	human 1 (SEQ. ID NO:45)	human 21/28'CL	RH V_H 21.6	Comment
102	112	CDR3	Y	Y	Y	Y	Y	
103	113	FR4	W	W	W	W	W	
104	114	|	G	G	G	G	G	
105	115	|	Q	Q	Q	Q	Q	
106	116	|	G	G	G	G	G	
107	117	|	T	T	T	T	T	
108	118	|	S	X	L	L	L	
109	119	|	V	V	V	V	V	
110	120	|	T	T	T	T	T	
111	121	|	V	V	V	V	V	
112	122	|	S	S	S	S	S	
113	123	FR4	S	S	S	S	S	

Legend: (Kabat) numbering according to Kabat et al., supra; (#) sequential numbering as used in the molecular modelling; (mouse 21.6) amino acid sequence of the V_H region from mouse 21.6 antibody; (mouse 2c) consensus sequence of mouse V_H regions from subgroup 2c (Kabat et al., supra); (human 1) consensus sequence of human V_H regions from subgroup 1 (Kabat et al., supra); (human 21/28'CL) amino acid sequence of a human V_H region (Dersimonian et al. (1987), supra); (RH V_H 21.6) amino acid sequence of version H1 of reshaped human 21.6 V_H region; (*) residues that are part of the canonical structures for the CDR loops (Chothia et al., supra); (underlined) residues in the human FRs where the amino acid residue was changed.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i i i) NUMBER OF SEQUENCES: 45

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 483 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(i x) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 53..430

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```
ATGAGGGCCC CTGCTCAGAT TTTTGGATTC TTGGTCAGGA GACGTTGTAG AA ATG          55
                                                          Met
                                                           1

AGA CCG TCT ATT CAG TTC CTG GGG CTC TTG TTG TTC TGG CTT CAT GGT        103
Arg Pro Ser Ile Gln Phe Leu Gly Leu Leu Leu Phe Trp Leu His Gly
      5                  10                  15

GCT CAG TGT GAC ATC CAG ATG ACA CAG TCT CCA TCC TCA CTG TCT GCA        151
Ala Gln Cys Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala
  20                  25                  30

TCT CTG GGA GGC AAA GTC ACC ATC ACT TGC AAG ACA AGC CAA GAC ATT        199
Ser Leu Gly Gly Lys Val Thr Ile Thr Cys Lys Thr Ser Gln Asp Ile
  35                  40                  45

AAC AAG TAT ATG GCT TGG TAC CAA CAC AAG CCT GGA AAA CGT CCT AGG        247
Asn Lys Tyr Met Ala Trp Tyr Gln His Lys Pro Gly Lys Arg Pro Arg
  50                  55                  60                  65

CTG CTC ATA CAT TAC ACA TCT GCA TTA CAG CCA GGC ATC CCA TCA AGG        295
Leu Leu Ile His Tyr Thr Ser Ala Leu Gln Pro Gly Ile Pro Ser Arg
                  70                  75                  80
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TTC AGT GGA AGT GGG TCT GGG AGA GAT TAT TCC TTC AAC ATC AGC AAC          343
Phe Ser Gly Ser Gly Ser Gly Arg Asp Tyr Ser Phe Asn Ile Ser Asn
                85                  90                  95

CTG GAG CCT GAA GAT ATT GCA ACT TAT TAT TGT CTA CAG TAT GAT AAT          391
Leu Glu Pro Glu Asp Ile Ala Thr Tyr Tyr Cys Leu Gln Tyr Asp Asn
        100                 105                 110

CTG TGG ACG TTC GGT GGA GGC ACC AAG CTG GAA ATC AAA CGGGCTGATG           440
Leu Trp Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys
    115                 120                 125

CTGCACCAAC TGTATCCATC TTCCCACCAT CCACCCGGGA TCC                          483
```

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 126 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: protein

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
Met Arg Pro Ser Ile Gln Phe Leu Gly Leu Leu Leu Phe Trp Leu His
 1               5                  10                  15

Gly Ala Gln Cys Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser
            20                  25                  30

Ala Ser Leu Gly Gly Lys Val Thr Ile Thr Cys Lys Thr Ser Gln Asp
        35                  40                  45

Ile Asn Lys Tyr Met Ala Trp Tyr Gln His Lys Pro Gly Lys Arg Pro
    50                  55                  60

Arg Leu Leu Ile His Tyr Thr Ser Ala Leu Gln Pro Gly Ile Pro Ser
65                  70                  75                  80

Arg Phe Ser Gly Ser Gly Ser Gly Arg Asp Tyr Ser Phe Asn Ile Ser
                85                  90                  95

Asn Leu Glu Pro Gln Asp Ile Ala Thr Tyr Tyr Cys Leu Gln Tyr Asp
            100                 105                 110

Asn Leu Trp Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys
        115                 120                 125
```

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 470 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(i x) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..420

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```
ATG AAA TGC AGC TGG GTC ATG TTC TTC CTG ATG GCA GTG GTT ACA GGG           48
Met Lys Cys Ser Trp Val Met Phe Phe Leu Met Ala Val Val Thr Gly
 1               5                  10                  15

GTC AAT TCA GAG GTT CAG CTG CAG CAG TCT GGG GCA GAG CTT GTG AAG           96
Val Asn Ser Glu Val Gln Leu Gln Gln Ser Gly Ala Glu Leu Val Lys
            20                  25                  30

CCA GGG GCC TCA GTC AAG TTG TCC TGC ACA GCT TCT GGC TTC AAC ATT          144
Pro Gly Ala Ser Val Lys Leu Ser Cys Thr Ala Ser Gly Phe Asn Ile
        35                  40                  45

AAA GAC ACC TAT ATA CAC TGT GTG AAG CAG AGG CCT GAA CAG GGC CTG          192
```

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```
Lys Asp Thr Tyr Ile His Cys Val Lys Gln Arg Pro Glu Gln Gly Leu
 50                  55                  60

GAG TGG ATT GGA AGG ATT GAT CCT GCG AAT GGT TAT ACT AAA TAT GAC   240
Glu Trp Ile Gly Arg Ile Asp Pro Ala Asn Gly Tyr Thr Lys Tyr Asp
 65                  70                  75                  80

CCG AAG TTC CAG GGC AAG GCC ACT ATA ACA GCT GAC ACA TCC TCC AAC   288
Pro Lys Phe Gln Gly Lys Ala Thr Ile Thr Ala Asp Thr Ser Ser Asn
                 85                  90                  95

ACA GCC TAC CTG CAG CTC AGC AGC CTG ACA TCT GAG GAC ACT GCC GTC   336
Thr Ala Tyr Leu Gln Leu Ser Ser Leu Thr Ser Glu Asp Thr Ala Val
            100                 105                 110

TAT TTC TGT GCT AGA GAG GGA TAT TAT GGT AAC TAC GGG GTC TAT GCT   384
Tyr Phe Cys Ala Arg Glu Gly Tyr Tyr Gly Asn Tyr Gly Val Tyr Ala
        115                 120                 125

ATG GAC TAC TGG GGT CAA GGA ACC TCA GTC ACC GTC TCCTCAGCCA        430
Met Asp Tyr Trp Gly Gln Gly Thr Ser Val Thr Val
    130                 135                 140

AAACGACACC CCCATCTGTC TATCCACTGG CCCGGGATCC                       470
```

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 140 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: protein

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```
Met Lys Cys Ser Trp Val Met Phe Phe Leu Met Ala Val Val Thr Gly
 1                   5                  10                  15

Val Asn Ser Glu Val Gln Leu Gln Gln Ser Gly Ala Glu Leu Val Lys
            20                  25                  30

Pro Gly Ala Ser Val Lys Leu Ser Cys Thr Ala Ser Gly Phe Asn Ile
        35                  40                  45

Lys Asp Thr Tyr Ile His Cys Val Lys Gln Arg Pro Glu Gln Gly Leu
    50                  55                  60

Glu Trp Ile Gly Arg Ile Asp Pro Ala Asn Gly Tyr Thr Lys Tyr Asp
 65                  70                  75                  80

Pro Lys Phe Gln Gly Lys Ala Thr Ile Thr Ala Asp Thr Ser Ser Asn
                 85                  90                  95

Thr Ala Tyr Leu Gln Leu Ser Ser Leu Thr Ser Glu Asp Thr Ala Val
            100                 105                 110

Tyr Phe Cys Ala Arg Glu Gly Tyr Tyr Gly Asn Tyr Gly Val Tyr Ala
        115                 120                 125

Met Asp Tyr Trp Gly Gln Gly Thr Ser Val Thr Val
    130                 135                 140
```

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 106 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: protein

(x i) SEQUENCE DESCRIPTION: SEQ ID NO 5:

```
Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Leu Gly
 1                   5                  10                  15
```

-continued

```
Gly Lys Val Thr Ile Thr Cys Lys Thr Ser Gln Asp Ile Asn Lys Tyr
            20                  25                  30

Met Ala Trp Tyr Gln His Lys Pro Gly Lys Arg Pro Arg Leu Leu Ile
        35                  40                  45

His Tyr Thr Ser Ala Leu Gln Pro Gly Ile Pro Ser Arg Phe Ser Gly
    50                  55                  60

Ser Gly Ser Gly Arg Asp Tyr Ser Phe Asn Ile Ser Asn Leu Glu Pro
65                  70                  75                  80

Glu Asp Ile Ala Thr Tyr Tyr Cys Leu Gln Tyr Asp Asn Leu Trp Thr
                85                  90                  95

Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys
            100                 105
```

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 107 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: protein

(x i) SEQUENCE DESCRIPTION: SEQ ID NO.6:

```
Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
1               5                   10                  15

Asp Arg Val Thr Ile Thr Cys Gln Ala Ser Gln Asp Ile Ile Lys Tyr
            20                  25                  30

Leu Asn Trp Tyr Gln Gln Thr Pro Gly Lys Ala Pro Lys Leu Leu Ile
        35                  40                  45

Tyr Glu Ala Ser Asn Leu Gln Ala Gly Val Pro Ser Arg Phe Ser Gly
    50                  55                  60

Ser Gly Ser Gly Thr Asp Tyr Thr Phe Thr Ile Ser Ser Leu Gln Pro
65                  70                  75                  80

Glu Asp Ile Ala Thr Tyr Tyr Cys Gln Gln Tyr Gln Ser Leu Pro Tyr
                85                  90                  95

Thr Phe Gly Gln Gly Thr Lys Leu Gln Ile Thr
            100                 105
```

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 106 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: protein

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:7.

```
Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
1               5                   10                  15

Asp Arg Val Thr Ile Thr Cys Lys Thr Ser Gln Asp Ile Asn Lys Tyr
            20                  25                  30

Met Ala Trp Tyr Gln Gln Thr Pro Gly Lys Ala Pro Arg Leu Leu Ile
        35                  40                  45

His Tyr Thr Ser Ala Leu Gln Pro Gly Ile Pro Ser Arg Phe Ser Gly
    50                  55                  60

Ser Gly Ser Gly Arg Asp Tyr Thr Phe Thr Ile Ser Ser Leu Gln Pro
65                  70                  75                  80
```

-continued

```
Glu Asp Ile Ala Thr Tyr Tyr Cys Leu Gln Tyr Asp Asn Leu Trp Thr
                85                  90                  95

Phe Gly Gln Gly Thr Lys Val Glu Ile Lys
            100                 105
```

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 107 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: protein

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:8.

```
Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
1               5                  10                  15

Asp Arg Val Thr Ile Thr Cys Gln Ala Ser Gln Asp Ile Ile Lys Tyr
            20                  25                  30

Leu Asn Trp Tyr Gln Gln Thr Pro Gly Lys Ala Pro Arg Leu Leu Ile
        35                  40                  45

Tyr Glu Ala Ser Asn Leu Gln Ala Gly Ile Pro Ser Arg Phe Ser Gly
    50                  55                  60

Ser Gly Ser Gly Arg Asp Tyr Thr Phe Thr Ile Ser Ser Leu Gln Pro
65                  70                  75                  80

Glu Asp Ile Ala Thr Tyr Tyr Cys Gln Gln Tyr Gln Ser Leu Pro Tyr
                85                  90                  95

Thr Phe Gly Gln Gly Thr Lys Leu Gln Ile Thr
            100                 105
```

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 123 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: protein

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:9:

```
Glu Val Gln Leu Gln Gln Ser Gly Ala Glu Leu Val Lys Pro Gly Ala
1               5                  10                  15

Ser Val Lys Leu Ser Cys Thr Ala Ser Gly Phe Asn Ile Lys Asp Thr
            20                  25                  30

Tyr Ile His Cys Val Lys Gln Arg Pro Glu Gln Gly Leu Glu Trp Ile
        35                  40                  45

Gly Arg Ile Asp Pro Ala Asn Gly Tyr Thr Lys Tyr Asp Pro Lys Phe
    50                  55                  60

Gln Gly Lys Ala Thr Ile Thr Ala Asp Thr Ser Ser Asn Thr Ala Tyr
65                  70                  75                  80

Leu Gln Leu Ser Ser Leu Thr Ser Glu Asp Thr Ala Val Tyr Phe Cys
                85                  90                  95

Ala Arg Glu Gly Tyr Tyr Gly Asn Tyr Gly Val Tyr Ala Met Asp Tyr
            100                 105                 110

Trp Gly Gln Gly Thr Ser Val Thr Val Ser Ser
        115                 120
```

(2) INFORMATION FOR SEQ ID NO:10:

-continued

(i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 119 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: protein

(x i) SEQUENCE DESCRIPTION: SEQ ID NO 10:

```
Gln Val Gln Leu Val Gln Ser Gly Ala Glu Val Lys Lys Pro Gly Ala
1               5                  10                  15

Ser Val Lys Val Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Ser Tyr
            20                  25                  30

Ala Met His Trp Val Arg Gln Ala Pro Gly Gln Arg Leu Glu Trp Met
        35                  40                  45

Gly Trp Ile Asn Ala Gly Asn Gly Asn Thr Lys Tyr Ser Gln Lys Phe
    50                  55                  60

Gln Gly Arg Val Thr Ile Thr Arg Asp Thr Ser Ala Ser Thr Ala Tyr
65                  70                  75                  80

Met Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr Tyr Cys
                85                  90                  95

Ala Arg Gly Gly Tyr Tyr Gly Ser Gly Ser Asn Tyr Trp Gly Gln Gly
            100                 105                 110

Thr Leu Val Thr Val Ser Ser
        115
```

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 123 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS. single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: protein

(x i) SEQUENCE DESCRIPTION: SEQ ID NO.11.

```
Gln Val Gln Leu Val Gln Ser Gly Ala Glu Val Lys Lys Pro Gly Ala
1               5                  10                  15

Ser Val Lys Val Ser Cys Lys Ala Ser Gly Phe Asn Ile Lys Asp Thr
            20                  25                  30

Tyr Ile His Trp Val Arg Gln Ala Pro Gly Gln Arg Leu Glu Trp Met
        35                  40                  45

Gly Arg Ile Asp Pro Ala Asn Gly Tyr Thr Lys Tyr Asp Pro Lys Phe
    50                  55                  60

Gln Gly Arg Val Thr Ile Thr Ala Asp Thr Ser Ala Ser Thr Ala Tyr
65                  70                  75                  80

Met Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr Tyr Cys
                85                  90                  95

Ala Arg Glu Gly Tyr Tyr Gly Asn Tyr Gly Val Tyr Ala Met Asp Tyr
            100                 105                 110

Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser
        115                 120
```

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 119 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS. single
(D) TOPOLOGY: linear

-continued

(i i) MOLECULE TYPE. protein

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Gln Val Gln Leu Val Gln Ser Gly Ala Glu Val Lys Lys Pro Gly Ala
1 5 10 15

Ser Val Lys Val Ser Cys Lys Ala Ser Gly Phe Asn Ile Lys Ser Tyr
20 25 30

Ala Met His Trp Val Arg Gln Ala Pro Gly Gln Gly Leu Glu Trp Met
35 40 45

Gly Trp Ile Asn Ala Gly Asn Gly Asn Thr Lys Tyr Ser Gln Lys Phe
50 55 60

Gln Gly Arg Val Thr Ile Thr Ala Asp Thr Ser Ala Ser Thr Ala Tyr
65 70 75 80

Met Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr Tyr Cys
85 90 95

Ala Arg Gly Gly Tyr Tyr Gly Ser Gly Ser Asn Tyr Trp Gly Glu Gly
100 105 110

Thr Leu Val Thr Val Ser Ser
115

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS.
(A) LENGTH: 119 ammo acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: protein

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:13.

Gln Val Gln Leu Val Gln Ser Gly Ala Glu Val Lys Lys Pro Gly Ala
1 5 10 15

Ser Val Lys Val Ser Cys Lys Ala Ser Gly Phe Asn Ile Lys Ser Tyr
20 25 30

Ala Met His Trp Val Arg Gln Ala Pro Gly Gln Arg Leu Glu Trp Met
35 40 45

Gly Trp Ile Asn Ala Gly Asn Gly Asn Thr Lys Tyr Ser Glu Lys Phe
50 55 60

Gln Gly Arg Val Thr Ile Thr Ala Asp Thr Ser Ala Ser Thr Ala Tyr
65 70 75 80

Met Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr Tyr Cys
85 90 95

Ala Arg Gly Gly Tyr Phe Gly Ser Gly Ser Asn Tyr Trp Gly Gln Gly
100 105 110

Thr Leu Val Thr Val Ser Ser
115

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 406 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE. cDNA

(i x) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION· 16..393

-continued

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:14·

```
AAGCTTGCCG CCACC ATG AGA CCG TCT ATT CAG TTC CTG GGG CTC TTG TTG        51
                 Met Arg Pro Ser Ile Gln Phe Leu Gly Leu Leu Leu
                   1               5                  10

TTC TGG CTT CAT GGT GCT CAG TGT GAC ATC CAG ATG ACA CAG TCT CCA        99
Phe Trp Leu His Gly Ala Gln Cys Asp Ile Gln Met Thr Gln Ser Pro
          15                  20                  25

TCC TCA CTG TCT GCA TCT CTG GGA GGC AAA GTC ACC ATC ACT TGC AAG       147
Ser Ser Leu Ser Ala Ser Leu Gly Gly Lys Val Thr Ile Thr Cys Lys
      30                  35                  40

ACA AGC CAA GAC ATT AAC AAG TAT ATG GCT TGG TAC CAA CAC AAG CCT       195
Thr Ser Gln Asp Ile Asn Lys Tyr Met Ala Trp Tyr Gln His Lys Pro
  45                  50                  55                  60

GGA AAA CGT CCT AGG CTG CTC ATA CAT TAC ACA TCT GCA TTA CAG CCA       243
Gly Lys Arg Pro Arg Leu Leu Ile His Tyr Thr Ser Ala Leu Gln Pro
                  65                  70                  75

GGC ATC CCA TCA AGG TTC AGT GGA AGT GGG TCT GGG AGA GAT TAT TCC       291
Gly Ile Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly Arg Asp Tyr Ser
              80                  85                  90

TTC AAC ATC AGC AAC CTG GAG CCT GAA GAT ATT GCA ACT TAT TAT TGT       339
Phe Asn Ile Ser Asn Leu Glu Pro Glu Asp Ile Ala Thr Tyr Tyr Cys
          95                 100                 105

CTA CAG TAT GAT AAT CTG TGG ACG TTC GGT GGA GGC ACC AAG CTG GAA       387
Leu Gln Tyr Asp Asn Leu Trp Thr Phe Gly Gly Gly Thr Lys Leu Glu
     110                 115                 120

ATC AAA CGTGAGTGGA TCC                                                406
Ile Lys
125
```

(2) INFORMATION FOR SEQ ID NO:15.

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 126 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE. protein

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:15:

```
Met Arg Pro Ser Ile Gln Phe Leu Gly Leu Leu Leu Phe Trp Leu His
  1               5                  10                  15

Gly Ala Gln Cys Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser
            20                  25                  30

Ala Ser Leu Gly Gly Lys Val Thr Ile Thr Cys Lys Thr Ser Gln Asp
        35                  40                  45

Ile Asn Lys Tyr Met Ala Trp Tyr Gln His Lys Pro Gly Lys Arg Pro
    50                  55                  60

Arg Leu Leu Ile His Tyr Thr Ser Ala Leu Gln Pro Gly Ile Pro Ser
65                  70                  75                  80

Arg Phe Ser Gly Ser Gly Ser Gly Arg Asp Tyr Ser Phe Asn Ile Ser
                85                  90                  95

Asn Leu Glu Pro Glu Asp Ile Ala Thr Tyr Tyr Cys Leu Gln Tyr Asp
            100                 105                 110

Asn Leu Trp Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys
        115                 120                 125
```

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 454 base pairs
(B) TYPE: nucleic acid

-continued

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(i x) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 16..441

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:16:

```
AAGCTTGCCG CCACC ATG GAC TGG ACC TGG CGC GTG TTT TGC CTG CTC GCC          51
                 Met Asp Trp Thr Trp Arg Val Phe Cys Leu Leu Ala
                   1               5                  10

GTG GCT CCT GGG GCC CAC AGC CAG GTG CAA CTA GTG CAG TCC GGC GCC          99
Val Ala Pro Gly Ala His Ser Gln Val Gln Leu Val Gln Ser Gly Ala
          15                  20                  25

GAA GTG AAG AAA CCC GGT GCT TCC GTG AAA GTC AGC TGT AAA GCT AGC         147
Glu Val Lys Lys Pro Gly Ala Ser Val Lys Val Ser Cys Lys Ala Ser
      30                  35                  40

GGT TTC AAC ATT AAA GAC ACC TAT ATA CAC TGG GTT AGA CAG GCC CCT         195
Gly Phe Asn Ile Lys Asp Thr Tyr Ile His Trp Val Arg Gln Ala Pro
  45                  50                  55                  60

GGC CAA AGG CTG GAG TGG ATG GGA AGG ATT GAT CCT GCG AAT GGT TAT         243
Gly Gln Arg Leu Glu Trp Met Gly Arg Ile Asp Pro Ala Asn Gly Tyr
                  65                  70                  75

ACT AAA TAT GAC CCG AAG TTC CAG GGC CGG GTC ACC ATC ACC GCA GAC         291
Thr Lys Tyr Asp Pro Lys Phe Gln Gly Arg Val Thr Ile Thr Ala Asp
              80                  85                  90

ACC TCT GCC AGC ACC GCC TAC ATG GAA CTG TCC AGC CTG CGC TCC GAG         339
Thr Ser Ala Ser Thr Ala Tyr Met Glu Leu Ser Ser Leu Arg Ser Glu
          95                 100                 105

GAC ACT GCA GTC TAC TAC TGC GCC AGA GAG GGA TAT TAT GGT AAC TAC         387
Asp Thr Ala Val Tyr Tyr Cys Ala Arg Glu Gly Tyr Tyr Gly Asn Tyr
      110                 115                 120

GGG GTC TAT GCT ATG GAC TAC TGG GGT CAA GGA ACC CTT GTC ACC GTC         435
Gly Val Tyr Ala Met Asp Tyr Trp Gly Gln Gly Thr Leu Val Thr Val
  125                 130                 135                 140

TCC TCA GGTGAGTGGA TCC                                                  454
Ser Ser
```

(2) INFORMATION FOR SEQ ID NO:17·

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 142 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: protein

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:17

```
Met Asp Trp Thr Trp Arg Val Phe Cys Leu Leu Ala Val Ala Pro Gly
  1               5                  10                  15

Ala His Ser Gln Val Gln Leu Val Gln Ser Gly Ala Glu Val Lys Lys
              20                  25                  30

Pro Gly Ala Ser Val Lys Val Ser Cys Lys Ala Ser Gly Phe Asn Ile
          35                  40                  45

Lys Asp Thr Tyr Ile His Trp Val Arg Gln Ala Pro Gly Gln Arg Leu
      50                  55                  60

Glu Trp Met Gly Arg Ile Asp Pro Ala Asn Gly Tyr Thr Lys Tyr Asp
  65                  70                  75                  80

Pro Lys Phe Gln Gly Arg Val Thr Ile Thr Ala Asp Thr Ser Ala Ser
                  85                  90                  95
```

-continued

```
Thr Ala Tyr Met Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Val
                100                 105                 110

Tyr Tyr Cys Ala Arg Glu Gly Tyr Tyr Gly Asn Tyr Gly Val Tyr Ala
            115                 120                 125

Met Asp Tyr Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser
    130                 135                 140
```

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 37 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (primer)

(x i) SEQUENCE DESCRIPTION SEQ ID NO:18:

```
CAGAAAGCTT GCCGCCACCA TGAGACCGTC TATTCAG                    37
```

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (primer)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:19:

```
CCGAGGATCC ACTCACGTTT GATTTCCAGC TTGGT                      35
```

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 37 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (primer)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:20:

```
CAGAAAGCTT GCCGCCACCA TGAAATGCAG CTGGGTC                    37
```

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (primer)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:21:

```
CCGAGGATCC ACTCACCTGA GCAGACGGTG ACT                        33
```

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (primer)

-continued

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:22:

GATGGTGACT CTATCTCCTA CAGATGCAGA CAGTGAGGA 39

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE. DNA (primer)

(x i) SEQUENCE DESCRIPTION. SEQ ID NO:23:

CTGTAGGAGA TAGAGTCACC ATCACTTGCA AG 32

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (primer)

(x i) SEQUENCE DESCRIPTION. SEQ ID NO:24:

AGGAGCTTTT CCAGGTGTCT GTTGGTACCA AGCCATATA 39

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 41 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY. linear

(i i) MOLECULE TYPE: DNA (primer)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:25·

ACCAACAGAC ACCTGGAAAA GCTCCTAGGC TGCTCATACA T 41

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 40 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (primer)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:26:

GCAGGCTGCT GATGGTGAAA GTATAATCTC TCCCAGACCC 40

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 42 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (primer)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:27:

ACTTTCACCA TCAGCAGCCT GCAGCCTGAA GATATTGCAA CT 42

-continued

(2) INFORMATION FOR SEQ ID NO 28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 59 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (primer)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:28:

CCGAGGATCC ACTCACGTTT CATTTCCACC TTGGTGCCTT GACCGAACGT CCACAGATT 59

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS.
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (primer)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO.29.

GGAAAAGCTC CTAGGCTGCT CATATATTAC ACA 33

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 38 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (primer)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:30:

CCGAGGATCC ACTCACGTTT GATTTCCACC TTTGTGCC 38

(2) INFORMATION FOR SEQ ID NO 31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 51 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (primer)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:31

AACCCAGTGT ATATAGGTGT CTTTAATGTT GAAACCGCTA GCTTTACAGC T 51

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 67 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (primer)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:32:

AAAGACACCT ATATACACTG GGTTAGACAG GCCCCTGGCC AAAGGCTGGA GTGGATGGGA 60

AGGATTG 67

(2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:

-continued

(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE. DNA (primer)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:33:

GACCCGGCCC TGGAACTTCG GGTCAT 26

(2) INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 66 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (primer)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:34:

GACCCGAAGT TCCAGGGCAG GGTCACCATC ACCGCAGACA CCTCTGCCAG CACCGCCTAC 60

ATGGAA 66

(2) INFORMATION FOR SEQ ID NO:35:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 64 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (primer)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:35:

CCATAGCATA GACCCCGTAG TTACCATAAT ATCCCTCTCT GGCGCAGTAG TAGACTGCAG 60

TGTC 64

(2) INFORMATION FOR SEQ ID NO 36:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 63 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS. single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (primer)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:36:

GGTAACTACG GGGTCTATGC TATGGACTAC TGGGGTCAAG GAACCCTTGT CACCGTCTCC 60

TCA 63

(2) INFORMATION FOR SEQ ID NO:37:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH. 37 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (primer)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:37:

CCAGGGCCGG GTCACCATCA CCAGAGACAC CTCTGCC 37

(2) INFORMATION FOR SEQ ID NO:38:

-continued

(i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (primer)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

CAGGCCCCTG GCCAAGGGCT GGAGTGG 27

(2) INFORMATION FOR SEQ ID NO:39.

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (primer)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:39

TACGCAAACC GCCTCTC 17

(2) INFORMATION FOR SEQ ID NO 40:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE. DNA (primer)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:

GAGTGCACCA TATGCGGT 18

(2) INFORMATION FOR SEQ ID NO:41.

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 116 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:

Gln Val Gln Leu Val Gln Ser Gly Ala Glu Val Lys Lys Pro Gly Ala
1 5 10 15

Ser Val Lys Val Ser Cys Lys Ala Ser Gly Tyr Ser Phe Thr Ser Tyr
20 25 30

Tyr Ile His Trp Val Arg Gln Ala Pro Gly Gln Gly Leu Glu Trp Val
35 40 45

Gly Tyr Ile Asp Pro Phe Asn Gly Gly Thr Ser Tyr Asn Gln Lys Phe
50 55 60

Lys Gly Lys Val Thr Met Thr Val Asp Thr Ser Thr Asn Thr Ala Tyr
65 70 75 80

Met Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr Tyr Cys
85 90 95

Ala Arg Gly Gly Asn Arg Phe Ala Tyr Trp Gly Gln Gly Thr Leu Val
100 105 110

Thr Val Ser Ser
115

-continued

(2) INFORMATION FOR SEQ ID NO:42:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 109 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS single
(D) TOPOLOGY linear

(i i) MOLECULE TYPE: protein

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:42:

```
Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Leu Gly
1               5                  10                  15

Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Gln Asp Asp Ile Ser Asn
            20                  25                  30

Tyr Leu Asn Trp Tyr Gln Gln Lys Pro Gly Gly Ser Pro Lys Leu Leu
        35                  40                  45

Ile Tyr Tyr Ala Ser Arg Leu His Ser Gly Val Pro Ser Arg Phe Ser
    50                  55                  60

Gly Ser Gly Ser Gly Thr Asp Tyr Ser Leu Thr Ile Ser Asn Leu Glu
65                  70                  75                  80

Gln Glu Asp Ile Ala Thr Tyr Phe Cys Gln Gln Gly Asn Thr Leu Pro
                85                  90                  95

Pro Arg Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys
            100                 105
```

(2) INFORMATION FOR SEQ ID NO:43:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 114 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: protein

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:43:

```
Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
1               5                  10                  15

Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Gln Asp Ser Leu Val Xaa
            20                  25                  30

Xaa Ser Ile Ser Asn Tyr Leu Asn Trp Tyr Gln Gln Lys Pro Gly Lys
        35                  40                  45

Ala Pro Lys Leu Leu Ile Tyr Ala Ala Ser Ser Leu Glu Ser Gly Val
    50                  55                  60

Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr
65                  70                  75                  80

Ile Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln
                85                  90                  95

Tyr Asn Ser Leu Pro Glu Trp Thr Phe Gly Gln Gly Thr Lys Val Glu
            100                 105                 110

Ile Lys
```

(2) INFORMATION FOR SEQ ID NO:44:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 125 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: protein

-continued

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:44:

```
Glu Val Gln Leu Gln Gln Ser Gly Ala Glu Leu Val Lys Pro Gly Ala
1               5                  10                  15

Ser Val Lys Leu Ser Cys Thr Ala Ser Gly Phe Asn Ile Lys Asp Thr
            20                  25                  30

Tyr Met His Trp Val Lys Gln Arg Pro Glu Gln Gly Leu Glu Trp Ile
        35                  40                  45

Gly Arg Ile Asp Pro Ala Asn Gly Asn Thr Lys Tyr Asp Pro Lys Phe
    50                  55                  60

Gln Gly Lys Ala Thr Ile Thr Ala Asp Thr Ser Ser Asn Thr Ala Tyr
65                  70                  75                  80

Leu Gln Leu Ser Ser Leu Thr Ser Glu Asp Thr Ala Val Tyr Tyr Cys
                85                  90                  95

Ala Arg Gly Tyr Tyr Tyr Tyr Asp Ser Xaa Val Gly Tyr Tyr Ala Met
            100                 105                 110

Asp Tyr Trp Gly Gln Gly Thr Xaa Val Thr Val Ser Ser
        115                 120                 125
```

(2) INFORMATION FOR SEQ ID NO:45:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 129 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: protein

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:45:

```
Gln Val Gln Leu Val Glu Ser Gly Ala Glu Val Lys Lys Pro Gly Ala
1               5                  10                  15

Ser Val Lys Val Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Ser Tyr
            20                  25                  30

Ala Ile Ser Trp Val Arg Gln Ala Pro Gly Gln Gly Leu Glu Trp Met
        35                  40                  45

Gly Trp Ile Asn Pro Tyr Gly Asn Gly Asp Thr Asn Tyr Ala Gln Lys
    50                  55                  60

Phe Gln Gly Arg Val Thr Ile Thr Ala Asp Thr Ser Thr Ser Thr Ala
65                  70                  75                  80

Tyr Met Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr Tyr
                85                  90                  95

Cys Ala Arg Ala Pro Gly Tyr Gly Ser Gly Gly Gly Cys Tyr Arg Gly Asp
            100                 105                 110

Tyr Xaa Phe Asp Tyr Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser
        115                 120                 125
```

What is claimed is:

1. A humanized immunoglobulin comprising a humanized heavy chain and a humanized light chain:

(1) the humanized light chain comprising three complementarity determining regions (CDR1, CDR2 and CDR3) having amino acid sequences from the corresponding complementarity determining regions of the mouse 21-6 immunoglobulin light chain variable domain designated SEQ. ID. No. 2, and a variable region framework from a human kappa light chain variable region framework sequence provided that at least one position selected from a first group consisting of L45, L49, L58 and L69 (Kabat numbering convention) is occupied by the same amino acid residue present in the equivalent position of the mouse 21-6 immunoglobulin light chain variable region framework; and

(2) the humanized heavy chain comprising three complementarity determining regions (CDR1, CDR2 and CDR3) having amino acid sequences from the corresponding complementarity determining regions of the mouse 21-6 immunoglobulin heavy chain variable domain designated SEQ. ID. No. 4, and a variable region framework from a human heavy chain variable region framework sequence provided that at least one position selected from a second group consisting of H27, H28, H29, H30, H44, H71 (Kabat numbering

convention) is occupied by the same amino acid residue present in the equivalent position of the mouse 21-6 immunoglobulin heavy chain variable region framework;

wherein the humanized immunoglobulin specifically binds to alpha-4 integrin with a binding affinity having a lower limit of about 10^7M^{-1} and an upper limit of about five-times the binding affinity of the mouse 21-6 immunoglobulin wherein the 21-6 immunoglobulin has the light chain with a variable domain designated SEQ ID NO: 2 and IgG1 heavy chain with a variable domain designated SEQ ID NO: 4.

2. The humanized immunoglobulin of claim **1** wherein the humanized light chain variable region framework is from an RE1 variable region framework sequence (SEQ. ID No:6) provided that at least one position is selected from the first group, and provided that at least one position selected from a third group consisting of positions L104, L105 and L107 (Kabat numbering convention) is occupied by the same amino acid residue present in the equivalent position of a kappa light chain from any human immunoglobulin other than RE1 (SEQ. ID No:6).

3. The humanized immunoglobulin of claim **2**, wherein the humanized heavy chain variable region framework is from a 21/28'CL variable region framework sequence (SEQ. ID No:10).

4. The humanized immunoglobulin of claim **3**, wherein the humanized light chain variable region framework comprises at least three amino acids from the mouse 21.6 immunoglobulin at positions in the first group and three amino acids from the kappa light chain from the human immunoglobulin other than REI at positions in the third group, and the humanized heavy chain variable region framework comprises at least five amino acids from the mouse 21.6 immunoglobulin at positions in the second group.

5. The humanized immunoglobulin of claim **4**, wherein the humanized light chain variable region framework is identical to the RE1 light chain variable region framework sequence except for the at least three positions from the first group and the three positions from the third group, and the heavy chain variable region framework is identical to the 21/28'CL heavy chain variable region framework sequence (SEQ. ID No:10) except for the at least five positions from the second group.

6. The humanized immunoglobulin of claim **5**, wherein the at least three positions from the first group are positions L45, L58 and L69, and at the least five positions from the second group are positions H27, H28, H29, H30 and H71.

7. The humanized immunoglobulin of claim **6**, wherein the humanized light chain comprises complementarity determining regions that are identical to the corresponding complementarity determining regions of the mouse 21-6 heavy chain, and the humanized heavy chain comprises complementarity determining regions that are identical to the corresponding complementarity determining regions of the mouse 21-6 heavy chain, except that the CDR3 region of the humanized heavy chain may or may not comprise a phenylalanine residue at position H98.

8. The humanized immunoglobulin of claim **7**, wherein the CDR3 of the humanized heavy chain comprises a phenylalanine residue at position H98.

9. The humanized immunoglobulin of claim **1**, wherein the amino acid sequence of the mature light chain variable region is the sequence designated La (SEQ. ID NO:7) in FIG. **6** (SEQ ID NO: 7).

10. The humanized immunoglobulin of claim **1**, wherein the amino acid sequence of the mature light chain variable region is the sequence designated Lb (SEQ. ID NO:8) in FIG. **6** (SEQ ID NO: 8).

11. The humanized immunoglobulin of claim **1**, wherein the amino acid sequence of the mature heavy chain variable region is the sequence designated Ha (SEQ. ID NO:11) in FIG. 7 (SEQ ID NO: 11).

12. The humanized immunoglobulin of claim **1**, wherein the amino acid sequence of the mature heavy chain variable region is the sequence designated Hb (SEQ. ID NO: 12) in FIG. 7 (SEQ ID NO: 12).

13. The humanized immunoglobulin of claim **1**, wherein the amino acid sequence of the mature heavy chain variable region is the sequence designated Hc (SEQ. ID NO:13) in FIG. 7 (SEQ ID NO: 13).

14. The humanized immunoglobulin of claim **9**, wherein the amino acid sequence of the mature heavy chain variable region is Ha (SEQ. ID NO:11) in FIG. 7 (SEQ ID NO: 11).

15. The humanized immunoglobulin of claim **9**, wherein the amino acid sequence of the mature heavy chain variable region is Hb (SEQ. ID NO:12) in FIG. 7 (SEQ ID NO: 12).

16. The humanized immunoglobulin of claim **9**, wherein the amino acid sequence of the mature heavy chain variable region is designated Hc (SEQ. ID NO:13) in FIG. 7 (SEQ ID NO: 13).

17. An antigen-specific binding fragment of the humanized immunoglobulin of claim **14** or claim **16**.

18. A humanized immunoglobulin of claim **14** or **16** that has a constant region domain.

19. A humanized immunoglobulin of claim **18** wherein the constant region domain is incapable of complement fixation and antibody dependent cellular toxicity.

20. The humanized immunoglobulin of claim **18**, wherein the effector function is capable of complement fixation or antibody dependent cellular toxicity.

21. A nucleic acid encoding a heavy chain of a humanized antibody of claim **1** or a binding fragment thereof.

22. A nucleic acid encoding a light chain of a humanized antibody of claim **1** or a binding fragment thereof.

23. A pharmaceutical composition comprising a humanized immunoglobulin of claim **14** or **16**, or a binding fragment thereof, and a pharmaceutically acceptable carrier therefor.

24. A method for detecting alpha-4 integrin, the method comprising:

contacting a humanized immunoglobulin of claim **14** or **16**, or a binding fragment thereof, to a tissue sample from a patient; and

detecting complexes formed by specific binding between the antibody or fragment and alpha-4 integrin present in the target sample.

25. A method of inhibiting adhesion of a leukocyte to an endothelial cell, the method comprising administering a therapeutically effective amount of the pharmaceutical composition of claim **23**.

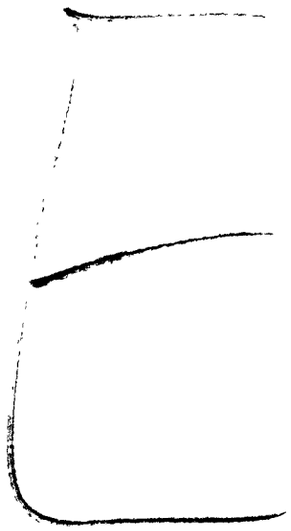
26. The method of claim **25**, wherein the endothelial cell is a brain cell.

27. A method of treating an inflammatory disease in a patient comprising administering to the patient a therapeutically effective amount of the pharmaceutical composition of claim **23**.

28. The method of claim **27** wherein the inflammatory disease is multiple sclerosis.

29. The method of claim **27**, wherein the patient is already suffering from multiple sclerosis and the administration of the pharmaceutical composition at least partially arrests the symptoms of the disease.

* * * * *



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The data shown below is from the records of the Patent and Trademark Office. If the maintenance fees and any necessary surcharges have been timely paid for the patents listed below, the notation "PAID" will appear in column 10, "STAT", below.

If a maintenance fee payment is defective, the reason is indicated by code in column 10, "STAT" below. TIMELY CORRECTION IS REQUIRED IN ORDER TO AVOID EXPIRATION OF THE PATENT. NOTE 37 CFR 1.377. THE PAYMENT(S) WILL BE ENTERED UPON RECEIPT OF ACCEPTABLE CORRECTION. IF PAYMENT OR CORRECTION IS SUBMITTED DURING THE GRACE PERIOD, A SURCHARGE IS ALSO REQUIRED. NOTE 37 CFR 1.20(k) and (l).

If the statement of small entity status is defective the reason is indicated below in column 10 for the related patent number. THE STATEMENT OF SMALL ENTITY STATUS WILL BE ENTERED UPON RECEIPT OF ACCEPTABLE CORRECTION.

PATENT NUMBER	FEE CODE	FEE AMT	SUR CHARGE	APPLICATION NUMBER	PATENT DATE	FILE DATE	PAY YR	SML ENT	STAT	ATTY DKT NUM
5,840,299	283	$440.00	$0.00	08/561,521	11/24/98	11/21/95	04	NO	PAID	15270-001420

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Brief Description of Activities during Regulatory Review Period for Tysabri®

October 23, 1996	BB-IND 6895 filed with FDA
October 24, 1996	BB-IND 6895 received at FDA
November 23, 1996	BB-IND 6895 in effect
February 13, 1997	Protocol Amendment: New Protocol Study AN100226-202
March 18, 1997	Teleconference with FDA to discuss AN100226-202 protocol
May 8, 1997	Teleconference with FDA to discuss AN100226-200 report format prior to submission of report.
June 18, 1997	Submission of safety monitoring committee guidelines for study AN 100226-202
July 11, 1997	Response to FDA Request for Information (Status update on AN100226-202 MRI SOPs, recent safety information, and confirmation/ list of attendees for the upcoming FDA teleconference.)
August 4, 1997	Information Amendment: Clinical Final Clinical Study0 Report filed for AN100226-101
October 20, 1997	Protocol Amendment: New Protocol Study AN100226-221

Brief Description of Activities during Regulatory Review Period for Tysabri®

January 20, 1998	Information Amendment: Pharm/Tox Final Report: Tox Study FRC 816, An Acute Toxicity Study (limit test) of AN100226 in mice
January 30, 1998	Information Amendment: Clinical Final Clinical Study Report filed for AN100226-200
February 03, 1998	Information Amendment: Pharm/Tox Final Report IM342, Tissue Cross-Reactivity
February 10, 1998	Annual Report: October 24, 1996 through November 9, 1997
February 26, 1998	Information Amendment: Pharm/Tox Final Report AL302, Embryofetal Development
April 03, 1998	Request for Telecon to discuss comparability package; included summary of strategy and rationale for nonclinical study design.
April 24, 1998	Information Amendment: Clinical, Pharm/Tox For April 29, 1998 meeting: comparability review, including a summary of the natalizumab development plan (acute & chronic) and 6 month monkey study protocol to support chronic.
April 29, 1998	Teleconference to discuss comparability program.
April 30, 1998	General Correspondence (FAX) Meeting confirmation for a request for a Pre-PLA meeting with CBER.
July 7, 1998	Information Amendment: Clinical Clinical Development Plan for MS and IBD

Brief Description of Activities during Regulatory Review Period for Tysabri®

August 05, 1998	Information Amendment: Pharm/Tox Final Reports 723-012-98 and AL300, single dose PK in monkeys
August 14, 1998	Request for Meeting Clinical Development Plan Discussion with FDA
October 19, 1998	FDA Internal Meeting Minutes (phone) Teleconference to discuss the natalizumab clinical development plan (submissions 052, 061, 062)
October 30, 1998	Protocol Amendment: New Protocol Study AN100226-224
December 30, 1998	General Correspondence Internal meeting minutes from FDA regarding clinical development plan.
January 19, 1999	General Correspondence: IND ownership change Letter from Athena to FDA, notification of transfer of natalizumab to Elan.
January 19, 1999	General Correspondence: IND ownership Change Letter from Elan to FDA, accepting ownership of natalizumab IND from Athena.
January 28, 1999	Annual Report IND Annual Report, Reporting period Nov. 10, 1997 through Nov. 09, 1998 including revised IB.
January 29, 1999	FDA Letter of Acknowledgement Letter from FDA acknowledging transfer of natalizumab from Athena to Elan.
February 1, 1999	Teleconference with FDA to discuss the powering of study AN100226-MS224

Brief Description of Activities during Regulatory Review Period for Tysabri®

April 23, 1999	Submission of Statistical Plan for Study AN100226-202
May 7, 1999	Protocol Amendment: New Protocol Study AN100226-231
January 27, 2000	Annual Report Annual Report for November 10, 1998 through November 09, 1999.
March 15, 2000	Submission of additional data to extend the stability of natalizumab clinical trial materials.
March 16, 2000	Submission of 6-month IV toxicology study in monkeys
April 24, 2000	Teleconference with FDA to discuss expiration and stability testing of natalizumab
May 16, 2000	FDA request for summary of status of development program
June 1, 2000	Status of development program provided to FDA (summary of where each trial stands with regard to enrolment, etc.)
June 15, 2000	Information Amendment: Nonclinical Presentation of nonclinical strategy
July 13, 2000	Teleconference with FDA to discuss nonclinical development program
August 8, 2000	Submission of summary of agreements with FDA made during July 13 teleconference in addition to summary of updated nonclinical development plan.

Brief Description of Activities during Regulatory Review Period for Tysabri®

September 1, 2000	Submission providing reports for the independent joint Safety Monitoring Committee held on 06/28/2000 and 08/11/2000. Submission of available data from the ongoing Phase II chronic-dosing MS Study AN100226-231 and European Phase II IBD Study AN100226CD-202.
November 9, 2000	Statistical Analysis Plan filed for Study AN100226-231
February 5, 2001	Information Amendment: Clinical Final Clinical Study Report filed for AN100226-221
March 05, 2001	General Correspondence: Biogen as CRO Letter naming Biogen as CRO for MS and CMC programs.
March 09, 2001	Meeting Request: Meeting Package End of Phase II meeting request
March 19, 2001	End of Phase II Meeting Scheduling Confirmation (fax)
March 22, 2001	Information Amendment: Pharm/Tox Summary of the natalizumab nonclinical program; updated repro-tox program, carcinogenicity rationale.
May 03, 2001	Annual Report Annual report covering the period of 11/09/99 to 12/12/00. Changing future reporting periods to 12/13 through 12/12.
May 24, 2001	FDA Response MW Called Biogen to clarify requirements for fast track designation and accelerated approval.
June 14, 2001	Elan and FDA meet to discuss nonclinical combo-tox

Brief Description of Activities during Regulatory Review Period for Tysabri®

June 19, 2001	Protocol Amendment: New Protocol New Protocol, C-1801
June 21, 2001	FDA Response: Clinical FDA Meeting Minutes of the May 17, 2001 End of Phase II Meeting.
August 16, 2001	Protocol Amendment: New Protocol New Protocol, C-1802
September 21, 2001	Protocol Amendment – New Protocol and Response to FDA Request for Information New Final Protocol, C-1802
October 01, 2001	Information Amendment: Clinical/CMC Comparability data package.
January 31, 2002	Teleconference between Mercedes Serabian and Elan/BiiB to discuss carcinogenicity proposal
February 22, 2002	Annual Report Submission of Annual Report for reporting period December 13, 2000 to December 12, 2001.
May 01, 2002	Waiver to Conduct Pediatric Studies Submission to FDA requesting a full waiver from conducting Pediatric Clinical Trials for BB-IND 6895.
June 27, 2004	Information Amendment: CMC Comparability Commercial Comparability: Cross-reference submission of CMC for comparability proposal dated June 2002.
August 02, 2002	FDA Response (Letter) FDA's response granting full waiver from conducting pediatric studies for natalizumab for MS BB-IND 6895.
August 30, 2002	Information Amendment: Clinical Final Clinical Study Report filed for AN100226-224

Brief Description of Activities during Regulatory Review Period for Tysabri®

October 01, 2002	Protocol Amendment: New Protocol New Clinical Protocol, C-1803
October 10, 2002	Teleconference with FDA to discuss Comparability Proposal.
November 25, 2002	Annual Report Annual report for August 17,2001 through August 16, 2002
February 10, 2003	General Correspondence – Clinical White Paper presenting plans to establish comparability of natalizumab used in phase 3 clinical trials with the material manufactured for commercial use.
April 23, 2003	Protocol Amendment – New Protocol New Protocol, C-1804
May 30, 2003	Information Amendment – CALA Questionnaire (Biogen's submission of the CALA Questionnaire for the eBLA)
June 3, 2003	Submission of one-month stability data for commercial drug product
June 11, 2003	Teleconference with Wilson Bryan of FDA on C-1801/C-1802 Statistical Analysis Plans.
July 14, 2003	Submission of written response to items discussed during the June 11, 2003 teleconference regarding the Statistical Analysis Plans for C-1801 and C-1802
August 21, 2003	Submission of C-1801, C-1802 Statistical Analysis Plans

Brief Description of Activities during Regulatory Review Period for Tysabri®

September 8, 2003	Submission of revised C-1801, C-1802 Statistical Analysis Plans
October 24, 2003	Submission of Study Protocol C-1808 for Subjects completing protocols C-1801, C-1802, or C-1803.
December 9, 2003	Phone call with FDA to discuss CMC topics.
February 17, 2004	Pre-BLA Meeting
March 01, 2004	General Correspondence: CMC, Clinical Biogen response to FDA from discussion at the 17 February 2004 pre-BLA meeting.
March 16, 2004	Meeting minutes issued by FDA for Pre-BLA Meeting
April 01. 2004	General Correspondence: Non-clinical Submission of 6 non-clinical study reports (kinetics and fertility and early embryonic development)
May 24, 2004	Original Biologics License Application 125104 for natalizumab is filed
June 14, 2004	Meeting Request submitted to BB-IND 6895 Meeting Request to discuss proprietary name for natalizumab
June 22, 2004	Amendment 001 to BLA 125104 Correction of errors in the original BLA.
June 23, 2004	FDA letter acknowledging receipt of BLA 125104 and designation of priority review.

Brief Description of Activities during Regulatory Review Period for Tysabri®

July 19, 2004	Information package on proprietary name submitted to BB-IND 6895
July 20, 2004	General Correspondence to BB-IND 6895 Letter with proposal for public presentation of one-year MS data
July 29, 2004	Amendment 002 to BLA 125104 Submission of final CSR for C-1803 including data through week 12.
August 3, 2004	Amendment 003 to BLA 125104 Per request by Dr. Wilson Bryan, submission of additional information AE datasets for C-1801 and C-1802
August 4, 2004	General Correspondence to BB-INB 6895: Clinical Supplement to the July 19, 2004 appeal package and meeting request (Serial No. 335) providing additional information regarding the proposed proprietary name for natalizumab.
August 6, 2004	Day 74 letter of potential review issues received.
August 9, 2004	Amendment 004 to BLA 125104 Per request by Dr. Lloyd Johnson, information provided pertaining to an investigation at three clinical sites.
August 16, 2004	Amendment 005 to BLA 125104 Response to questions raised in the Day 74 letter.
August 19, 2004	Teleconference between Biogen Idec, Dr. Elena Gubina and Dr. Joseph Kutza to discuss questions regarding cell banks.
August 23, 2004	Amendment 006 to BLA 125104 Responses to 10 questions regarding immunogenicity assays that were raised by the CMC review team

Brief Description of Activities during Regulatory Review Period for Tysabri®

September 10, 2004	Amendment 007 to BLA 125104 Response to information request
September 10, 2004	Amendment 008 to BLA 125104 Completion of response to Day 74 Deficiency Letter
September 13, 2004	Amendment 009 to BLA 125104 Formal request for Accelerated Approval
September 15, 2004	Amendment 010 to BLA 125104 Responses to VCAM Lysate Assay questions
September 21, 2004	Amendment 011 to BLA 125104 Response to questions from Dr. Wilson Bryan about PK data/antibody parameters
September 22, 2004	Amendment 012 to BLA 125104 120-day safety update
September 23, 2004	Amendment 013 to BLA 125104 Responses to questions from Dr. Elena Gubina and Dr. Joseph Kutza during August 19, 2004 teleconference between Biogen Idec and FDA. Questions pertained to cell banks.
September 24, 2004	Teleconference with Dr. Lei Zhang and Dr. Steven Koslowski to discuss additional request for VCAM lysate potency data.
October 6, 2004	Amendment 014to BLA 125104 Response to questions about immunogenicity assay raised during the preapproval inspection of the Biogen Idec Oceanside facility on September 20, 2004.
October 7, 2004	Amendment 015 to BLA 125104 Response to request for drug substance manufacturing time raised during RTP inspection from August 23 – 27, 2004. Response to question about End of Production Cell Bank

	adventitious virus testing raised in Day 74 deficiency letter.
October 7, 2004	Amendment 016 to BLA 125104 Information requested by Dr. Bryan for PK data listings for C-1801 and C-1802
October 12, 2004	Amendment 017 to BLA 125104 Response to information request in a Sept 24, 2004 teleconference with Dr. Lei Zhang and Dr. Steven Koslowski.
October 13, 2004	Amendment 018 to BLA 125104 Per request during the RTP pre-approval inspection, revision of shipping validation to reflect that the drug product will be shipped at 2-8C and not 2-15C.
October 13, 2004	Amendment 019 to BLA 125104 Provision of updated Drug Product stability data.
October 15, 2004	Amendment 020 to BLA 125104 Provision of revised package insert based on the 120-day safety update.
October 26, 2004	Amendment 021 to BLA 125104 Response to questions from CMC reviewers
October 27, 2004	Amendment 022 to BLA 125104 Response to questions from CMC reviewers
November 1, 2004	Amendment 023 to BLA 125104 Final report for the evaluation of in vivo adventitious viruses from EPCB derived from Drug Substance batch LC3-02-009
November 3, 2004	Amendment 024 to BLA 125104 Response to CMC questions

Brief Description of Activities during Regulatory Review Period for Tysabri®

November 3, 2004	Amendment 025 to BLA 125104 Submission of Tysabri and Velascue Tradenames as alternatives for Antegrex.
November 5, 2004	Amendment 026 to BLA 125104 Response to CMC questions
November 5, 2004	Amendment 027 to BLA 125104 Response to request for information from Drs. Mahmood and Bryan. Clarification of Avonex's impact on natalizumab via PK values.
November 5, 2004	Amendment 028 to BLA 125104 Submission of revised package insert
November 9. 2004	Amendment 029 to BLA 125104 Response to CMC question regarding reference standard
November 11, 2004	Amendment 030 to BLA 125104 Revised container and vials labels
November 15, 2004	Amendment 031 to BLA 125104 Response to questions from clinical reviewer
November 15, 2004	Amendment 032 to BLA 125104 Response to questions from clinical reviewer
November 15, 2004	Amendment 033 to BLA 125104 Response to questions from clinical reviewer
November 15, 2004	Amendment 034 to BLA 125104 Response to questions from clinical reviewer

Brief Description of Activities during Regulatory Review Period for Tysabri®

November 15, 2004	Amendment 035 to BLA 125104 Response to questions from CMC reviewer regarding VCAM lysate potency assay
November 16, 2004	Amendment 036 to BLA 125104 Response to requests regarding draft label
November 19, 2004	Submission of Tysabri promotional materials for pre-clearance
November 19, 2004	Amendment 037 to BLA 125104 Response to post-marketing commitments
November 23, 2004	Amendment 038 to BLA 125104 Response to post-marketing commitments
November 23, 2004	Amendment 039 to BLA 125104 Submission of final draft label
November 23, 2004	FDA grants approval of Tysabri in MS: Approval Letter

AUTHORIZATION FOR ATHENA NEUROSCIENCES, INC. TO RELY ON REGULATORY REVIEW OF TYSABRI

Biogen Idec Inc. ("Biogen") submits this Authorization in support of an Application for Patent Term Extension, filed by Athena Neurosciences, Inc. ("Athena"), pursuant to 35 U.S.C. § 156, for United States Patent No. 5,840,299 ("the '299 patent").

WHEREAS, the '299 patent was assigned to Athena by virtue of an Assignment from Mary M. Bendig, Olivier J. Leger, Jose Saldanha, S. Tarran Jones, and Ted A. Yednock to Athena recorded in the U.S. Patent and Trademark Office ("PTO") at Reel 007883, Frame 0465 on April 8, 1996.

WHEREAS, on or about October 23, 1996, Athena filed an investigational new drug ("IND") application number BB-IND 6895 for natalizumab with the FDA. On or about July 1, 1996, Elan Pharmaceuticals, Inc. ("Elan") acquired Athena, including all rights to the IND application number BB-IND 6895. On or about January 19, 1999, Athena notified the FDA of the transfer of IND application number BB-IND 6895 to Elan.

WHEREAS, on or about August 15, 2000, Elan and Biogen, Inc. (predecessor in interest to Biogen Idec Inc.) entered into a collaboration agreement for the development of TYSABRI whereby Biogen was granted a license to the '299 patent. On or about March 5 2001, Elan notified the FDA that Biogen was to be recognized as the contract research organization ("CRO") and manufacturer of TYSABRI.

WHEREAS, on or about May 24, 2004, Biogen filed a biologics license application ("BLA") for TYSABRI. On November 23, 2004, Biogen received approval from the FDA to market TYSABRI for treatment of patients with relapsing forms of multiple sclerosis to reduce the frequency of clinical exacerbations.

WHEREAS, Biogen desires to extend the term of the '299 patent based on the FDA approval of TYSABRI in order to increase the term of patent coverage for TYSABRI.

NOW THEREFORE, the undersigned, whose title is supplied below and who is empowered to act on behalf of Biogen, hereby authorizes Athena to rely on the FDA regulatory review of TYSABRI (natalizumab) for purposes of obtaining a patent term extension for the '299 patent pursuant to 35 U.S.C. § 156. The undersigned further declares that all statements made

herein of the undersigned's own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity or enforceability of the '299 patent or of any patent term extension thereof.

Signed,

Date: January 18, 2005

Name: Thomas J. Bucknum
Title: Executive Vice President and General Counsel
Biogen Idec Inc.

AUTHORIZATION FOR ATHENA NEUROSCIENCES, INC. TO RELY ON REGULATORY REVIEW OF TYSABRI

Elan Pharmaceuticals, Inc. ("Elan") submits this Authorization in support of an Application for Patent Term Extension, filed by Athena Neurosciences, Inc. ("Athena"), pursuant to 35 U.S.C. § 156, for United States Patent No. 5,840,299 ("the '299 patent").

WHEREAS, the '299 patent was assigned to Athena by virtue of an Assignment from Mary M. Bendig, Olivier J. Leger, Jose Saldanha, S. Tarran Jones, and Ted A. Yednock to Athena recorded in the U.S. Patent and Trademark Office ("PTO") at Reel 007883, Frame 0465 on April 8, 1996, an Assignment from Ted A. Yednock to Athena recorded in the PTO at Reel 012090, Frame 0786 on December 4, 2000, and an Assignment from Ted A. Yednock to Athena recorded in the PTO at Reel 011306, Frame 0071 on November 14, 2000.

WHEREAS, on or about October 23, 1996, Athena filed an investigational new drug ("IND") application number BB-IND 6895 for natalizumab with the FDA. On or about July 1, 1996, Elan Pharmaceuticals, Inc. ("Elan") acquired Athena, including all rights to the IND application number BB-IND 6895. On or about January 19, 1999, Athena notified the FDA of the transfer of IND application number BB-IND 6895 to Elan.

WHEREAS, on or about August 15, 2000, Elan and Biogen, Inc. (predecessor in interest to Biogen Idec Inc.) entered into a collaboration agreement for the development of TYSABRI whereby Biogen was granted a license to the '299 patent. On or about March 5 2001, Elan notified the FDA that Biogen was to be recognized as the contract research organization ("CRO") and manufacturer of TYSABRI.

WHEREAS, on or about May 24, 2004, Biogen filed a biologics license application ("BLA") for TYSABRI. On November 23, 2004, Biogen received approval from the FDA to market TYSABRI for treatment of patients with relapsing forms of multiple sclerosis to reduce the frequency of clinical exacerbations.

WHEREAS, Elan desires to extend the term of the '299 patent based on the FDA approval of TYSABRI in order to increase the term of patent coverage for TYSABRI.

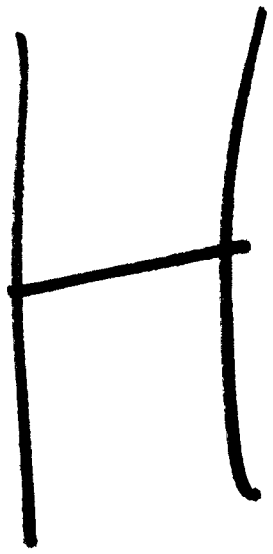
NOW THEREFORE, the undersigned, whose title is supplied below and who is empowered to act on behalf of Elan, hereby authorizes Athena to rely on the FDA regulatory review of TYSABRI (natalizumab) for purposes of obtaining a patent term extension for the '299

patent pursuant to 35 U.S.C. § 156. The undersigned further declares that all statements made herein of the undersigned's own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity or enforceability of the '299 patent or of any patent term extension thereof.

Signed,

Date: Jan. 14, 2005

Jack C. Laflin
EVP Global Core Services
Elan Pharmaceuticals Inc.



PATENT
Atty. Docket No.: 8576.0067

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re U.S. Patent No. 5,840,299)
)
Issued: November 24, 1998)
)
To: Mary S. Bendig, Olivier J. Léger, José Saldanha,)
S. Tarran Jones, Ted A. Yednock)
)
Assignee: Athena Neurosciences, Inc.)
)
For: HUMANIZED ANTIBODIES AGAINST LEUKOCYTE)
ADHESION MOLECULE VLA-4)

MAIL STOP PATENT EXT.
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Attachment H

Sir:

CERTIFICATION

I, CHARLES E. VAN HORN, do hereby certify that this accompanying application for extension of the term of U.S. Patent No. 5,840,299 under 35 U.S.C. § 156 including its attachments and supporting papers is being submitted as one original and two (2) copies thereof.

Respectfully submitted,

FINNEGAN, HENDERSON, FARABOW,
GARRETT & DUNNER, L.L.P.

Date: January 18, 2005

By: Charles E Van Horn
Charles E. Van Horn
Reg. No. 40,266